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THE ROLE OF THE THYROID IN KIDNEY DEVELOPMENT IN THE TADPOLE[1]

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INTRODUCTION

THE well-known experiments of Gudernatsch (1911, 1914) stimulated the investigation of thyroid function in amphibians. Since then the technique for thyroidectomy in tadpoles devised by Allen (1916) and Hoskins and Hoskins (1917), the use of thyroxin, and the administration of goitrogenic drugs such as thiourea and its derivatives have provided much information on growth, development, and metamorphic patterns in many anuran species (See review, Lynn and Wachowski, 1951).

Although many of the investigations on amphibian metamorphosis have been concerned with external metamorphic features, much attention has also been given to metamorphic changes exhibited by specific organs. In a recent study of the effects of the administration of thyroxin and thyroid-inhibiting drugs on the external and internal features of *Eleutherodactylus martinicensis*, an anuran species which lacks an aquatic larval stage (Lynn and Peadon, 1955), it was found that pronephric degeneration was inhibited by treatment with 0.05% thiourea or with 0.01 or 0.005% phenylthiourea while precocious development and rapid pronephric degeneration occurred in embryos treated with thyroxin (1 part in 10 million). These results suggest that in this species pronephric degeneration is controlled by thyroid activity, and prompt the question whether a similar condition prevails in anuran species which have an aquatic larval stage.

The origin and early development of the amphibian pronephros are well known (Field, 1891; Matsukura, 1935; Dalcq, 1942; van Gee-

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truyden, 1942; Holtfreter, 1944; and Cambar, 1949), but the later degeneration of the head kidney at metamorphosis has received little attention. A search of the literature reveals some rather brief accounts of the process (Field, 1891; Shore, 1901; Hall, 1904; Fraser, 1950; Jaffee, 1954), but detailed histological descriptions are lacking. Recently, Koszarek (1956) provided a somewhat fuller account of pronephric degeneration in *Rana pipiens,* an anuran species with an aquatic larval stage. However, a more detailed histological study of pronephric degeneration in a species of this type through a series of stages extending from the early tadpole stage through metamorphosis seems warranted.

In the present investigation, a histological study was made of stages of normal pronephric degeneration in *Rana sylvatica* and a correlation of these stages was made with the appearance of certain external metamorphic features. By the use of thiourea and by thyroxin administration, an attempt was also made to ascertain the possible role of the thyroid in pronephric degeneration.

Materials and Methods

For this study, frogs of the species *Rana sylvatica,* the common wood frog, were used. A total of approximately six hundred eggs, obtained from a Maryland pond in March, 1956, and March, 1957, was used. Shortly after hatching, the tadpoles were divided into groups of twenty, and placed in white enamel pans containing tap water. They were maintained at a room temperature of approximately 22°C, and the water was changed daily or every other day depending on its condition. Tadpoles were fed cooked spinach, egg yolk, and oatmeal flakes.

After preliminary experiments to ascertain the most favorable dosages and periods of treatment, the following procedure was adopted. Six groups of twenty animals each were placed in a solution of 0.03% thiourea in tap water. Treatment was initiated at various times from the hind limb bud stage to the 2 mm. hind limb stage. The tadpoles so treated grew rapidly while fore-limb protrusion and tail resorption were inhibited, and hind limb growth was markedly but incompletely inhibited. Treatment of the experimental animals was continued for approximately three and one-half months after completion of metamorphosis in controls.

Five groups of tadpoles were treated with thyroxin (1 part in 10

million). Tadpoles immersed in a solution of thyroxin at either the hind limb bud stage or the 1.5-3.0 mm. hind limb stage underwent very rapid metamorphic changes. Complete metamorphosis was not attained, however, for the tadpoles died after three to six days treatment. Within this short period, rapid growth of the hind limbs and resorption of the tail occurred, the fore-limbs appeared, and the tadpole mouth parts were lost. Day to day changes in pronephroi and other internal organs were followed in sectioned material. Control groups were maintained for all experiments.

Representative specimens from control and experimental groups were selected for fixation at frequent intervals. In 1956, control and thiourea specimens were preserved on an average of every three days during early metamorphic stages, and more frequently during periods when the controls were undergoing rapid metamorphic changes. In 1957, treatment of all animals began at the hind limb bud stage, and control and thiourea-treated specimens were preserved approximately every two days during early stages of metamorphosis and every three days toward metamorphic completion. Thyroxin-treated animals were preserved daily.

Specimens were fixed in equal parts of a saturated solution of $HgCl_2$ and ethyl alcohol (Cowdry, 1928) for a period of three to four days, washed from 18-24 hours in running tap water, and stored in 70% alcohol. Before embedding in paraffin, the following measurements were taken: body length, tail length, limb length, and maximum width. The animals were sectioned at 5 micra, and two staining procedures were employed: Mallory's triple stain (acid fuchsin, aniline blue, and orange G) and the Himes-Moriber (1956) triple stain for desoxyribosenucleic acid, polysaccharide, and protein. A preliminary series of control specimens from hatching through metamorphosis was sectioned to trace the complete history of the development and the degeneration of the pronephros. Seven stages from the hind limb bud stage through metamorphosis were arranged according to hind limb length and tail size for comparative studies of pronephric degeneration in control and thiourea-treated specimens. Three to seven animals from each of the seven stages were sectioned and studied. Pronephric degeneration in thiourea-treated specimens and controls was also studied using the number of days of treatment as a criterion of comparison. From three to eight animals in each of the five groups of thyroxin-treated specimens were sectioned, and day-to-

day changes in the pronephroi and other organs were compared with those of the controls to observe the course of pronephric degeneration during accelerated metamorphosis.

Detailed histological and cytological studies of sections were made with both the light and phase microscopes.

Observations and Results

A. *Development and Degeneration of the Pronephros in Untreated Tadpoles*

Although the early stages of pronephric growth and development, beginning with stage 20 (Pollister and Moore, 1937), were examined, detailed study was confined to animals 8 mm. in body length through animals that had undergone complete metamorphosis. Tail length and hind limb length were used as criteria in distinguishing the progressive series of metamorphic stages. Slight variations within a given stage were noted but, in general, the degenerative process followed a consistent pattern. Although a definite pronephric area persists until complete metamorphosis has taken place, the change from a fully functional pronephros to a definitely degenerated organ occurs rather early in metamorphosis. Evidences of degeneration may be seen almost simultaneously with the beginning of rapid growth of hind limbs, and the pronephros loses the characteristics of a functioning organ before the hind limbs have reached one-half their maximum size and before tail resorption and fore-limb protrusion occur.

1. *Origin and early development*

The paired pronephroi, which are derived from thickenings in the somatic wall of the nephrotomal region at the level of the second, third, and fourth somites, project laterally between the hypomere and the body ectoderm. When development is completed, they are massive, globular organs embedded in the dorsal wall of the body cavity. Several structures make up the functioning larval kidney. Three paired, transverse pronephric tubules, at the level of somites two, three, and four, open into the coelomic cavity by funnel-shaped, ciliated nephrostomes. Longitudinal fusion of nephrocoels on each side of the body results in the formation of common tubes, the paired pronephric or segmental ducts, which continue to grow posteriorly as far as the cloaca. The collecting tubules are continuous with and between the pronephric tubules and the segmental duct. Collecting

tubules are formed as a result of the extensive elongation and convolution of the pronephric tubules and the proximal part of the segmental duct. All the tubules are embedded in sinuses of the posterior cardinal veins, and the segmental duct, posterior to the pronephros, is accompanied by the posterior cardinal vein on the median side. A pronephric capsule of connective tissue, formed from somatic mesoderm and myotome, encloses the entire pronephros and the pronephric sinus of the posterior cardinal vein.

Although not directly connected with the pronephros, the glomus, which is a rudimentary capillary network bound by a peritoneal layer, is associated with the larval kidney by reason of its function and location, and by the fact that it degenerates simultaneously with the pronephros. The glomus develops from a horizontal fold of splanchnopleure. This forms a ridge directly across the body cavity from the pronephros, and extends from the first nephrostome to slightly beyond the third nephrostome. The glomus is attached to the dorsal aorta, and is suspended in the coelomic cavity.

2. *Early stage of complete functional activity*

Tadpoles at this stage showed the following features: body length, 8.5-9.0 mm.; maximum body width, 3.8-5.8 mm.; tail length, 22.7-13.8 mm.; hind limb buds. The pronephros in these animals is a large, fully functional organ. Its anterior end is definitely rounded in section, and consists of a loose arrangement of tubules (Figure 1). The loose, rounded appearance continues through the central sections, but the organ is more elliptical, in section, posteriorly.

The epithelial cells of the pronephric tubules are large, cuboidal cells with definite boundaries. A very prominent brush border is present. Secretions or waste products often obscure these long, coarse striations, and clumping of these materials is perhaps responsible for the irregular, peak-like form which is noted along the free border of the pronephric tubule cells (Figure 2). A thin but distinct basal membrane surrounds the tubules (Figure 3). A large, clear lumen containing sparse material, except along the brush border, is characteristic of this stage (Figure 2). The cytoplasm contains many granular inclusions, particularly just beneath the brush border. Nuclei, which are so large that they almost fill the cells, are centrally located, spherical, and have well-defined nuclear membranes (Figure 3). Many granular inclusions are contained within the nucleus,

and one or, in many instances, two large homogeneous nucleoli are present.

The lower, more rectangular epithelial cells of the collecting tubules are markedly lower than the epithelial cells of the pronephric tubule cells, and they have less definite cell boundaries (Figure 4). A smooth free surface and a very delicate basal membrane can be observed. Most of the lumina are relatively clear and large, but small amounts of material can be seen within the lumina, particularly in the posterior region of the organ. Collecting tubule cells have a highly granular cytoplasm, and a large, oval or spherical nucleus. Nuclei of these cells appear to have fewer nuclear inclusions, and frequently seem to take up the entire cell area, bulging into the lumen. One or two prominent nucleoli are present.

In serial sections, the segmental duct can be observed readily in the more posterior regions of the pronephros. The height of the epithelium, the appearance and position of the nucleus, the smooth lumen border, and the cytoplasmic inclusions are very similar to those of the epithelium of the collecting tubules. The segmental duct appears to arise toward the inner region of the organ adjacent to the pronephric capsule (Figure 5). It undergoes considerable twisting and coiling, within the pronephric area only, and is then drawn medially toward the dorsal aorta. More posteriorly, the segmental duct with surrounding blood cells and connective tissue capsule is separated from the remaining pronephric area, and becomes situated adjacent to and parallel with the dorsal aorta. This duct can be traced through many sections.

The nephrostomes are made up of cuboidal epithelium somewhat higher, but similar in other details to that of the collecting tubule (Figure 6). The nephrostome has a compact lining of long cilia.

At this stage, the intertubular sinuses of many specimens were large and free of cellular elements except for a small number of blood-forming cells. However, a few specimens showed more intertubular, cellular components in the region bordering the coelomic cavity, especially in the vicinity of the nephrostome. Pigment content varies slightly in specimens of the same stage, but all show interrupted, band-like aggregations of pigment in the mesenchymal tissue between the pronephric capsule and the epidermis. There are also thinly scattered, discrete granules in the pronephric tubule cells, and a greater number of granules toward the border of the lumen above the nucleus

in the collecting tubule cells (Figure 4) and the nephrostomes. There is no pigment in the intertubular sinuses or in the inner layer of the pronephric capsule.

The pronephric capsule appears as a very thin, delicate, non-pigmented membrane (Figure 1). A wide band of mesenchymal cells with pigment is present in the region of the integument, in close proximity to the pronephric capsule. A large glomus containing many capillaries and blood cells is attached to the dorsal aorta and suspended freely in the coelomic cavity (Figure 1). It appears that the growth of connective tissue in the region of the pronephros takes place anteriorly first. At this stage, at the most anterior region, a small amount of connective tissue intervenes between the dorsal aorta and the pronephros. Anteriorly, the developing lungs come very close to the pronephric capsule, almost sealing off the pronephric chamber. An increasingly wider space between the developing lungs and the pronephros is present in the central and posterior sections.

Sections stained with Himes-Moriber triple stain reveal that the bulk of the cell content, particularly in pronephric tubule cells, is protein in nature. Both the brush border and the nephrostomal cilia react positively to the protein-specific component of the stain. A narrow border lining both types of tubules is polysaccharide-positive. Material adhering to the brush borders or within the lumina generally reacts positively to the polysaccharide stain, but in some lumina, strands of protein-positive material were noted. Himes-Moriber stain indicates that the nuclei are rich in DNA, the nuclei staining green in the pronephric tubule cells and blue in the collecting tubule cells. The nucleoli, which are more prominent in the pronephric tubule cells, appear homogeneous, and give indication of being made up chiefly of protein material. It should be noted, however, that this stain as used does not differentiate RNA.

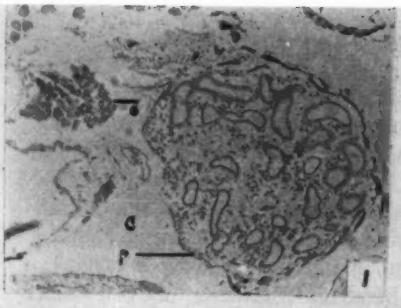
3. *Advanced stage of complete functional activity*

Measurements of tadpoles of this stage are: body length, 9.4-10.4 mm.; tail length, 14.0-19.0 mm.; hind limb length, 1.0-1.5 mm.; maximum width, 5.1-6.6 mm. The pronephric area is larger than in the preceding stage. Although the lumina continue to be large and relatively empty, most specimens show an increase of coagulated material along the brush borders and within the lumina (Figure 7). Therefore, a peak-like appearance at the lumen border of the pronephric

PLATE I

1. Anterior section through the pronephros of a control specimen at stage 1. X 50.
2. Section of the pronephric tubule showing brush border with coagulated material. X 950.
3. Section through a typical pronephric tubule. X 950.
4. Section through a typical collecting tubule. X 950.
5. Posterior section through the pronephros showing elongated collecting duct from which arises the segmental duct. X 50.
6. Section through a ciliated nephrostome opening into the coelom. X 475.
7. Anterior section through the pronephros of a control specimen at stage 2. Note glomus. X 50.
8. Section through a pronephric tubule showing a vacuole containing coagulated material. X 950.

B	brush border	L	lung
BC	blood cells	LN	lumen
BV	blood vessel	LV	liver
C	coelomic cavity	M	muscle fibers
CA	cartilage	ML	Mullerian duct
CN	connective tissue	N	nephrostome
CT	collecting tubule	P	pronephros
DA	dorsal aorta	PG	pigment
DP	degenerated pronephros	PT	pronephric tubule
G	glomus	R	excretory and secretory remnants
GC	glomerular cavity	S	sinus
I	intestine	V	vacuole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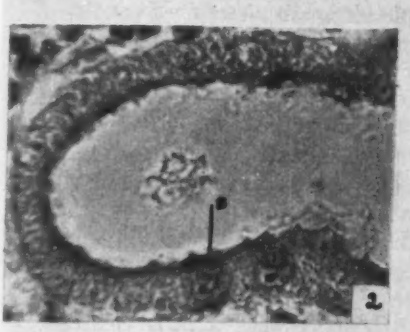
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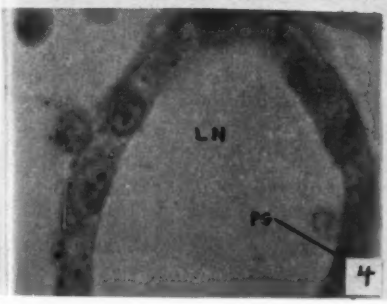
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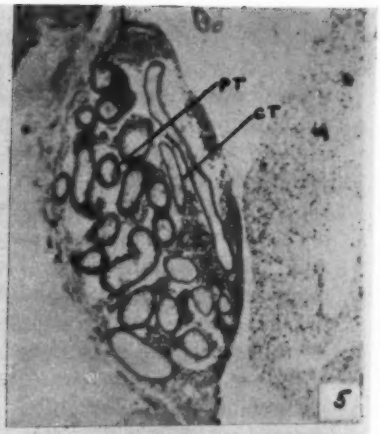
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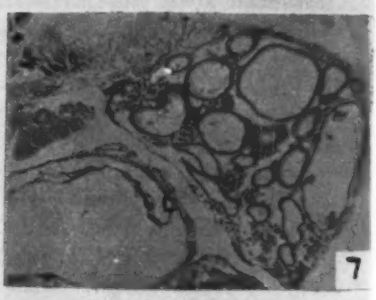
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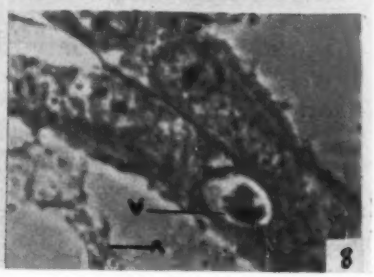
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tubule cells was frequently noted. As a result, those brush borders, not obscured by material, are particularly prominent. A few vacuoles, usually with discrete, stainable inclusions, are evident within the cytoplasm (Figure 8).

Very little variation from the preceding stage is seen in the collecting tubule cells. Difference in epithelial height between the pronephric and collecting tubule cells is now more marked. The very large lumina, the very low, cuboidal epithelium, and the indistinct cell membranes indicate that the collecting tubules are in a stretched condition. At this stage, the nuclei of collecting tubule cells are generally oval, and seem to contain fewer granular inclusions.

Cuboidal epithelium intermediate in height between that of the pronephric tubule cells and the collecting tubule cells characterizes the intact and functioning nephrostome. Spherical nuclei are located centrally or basally or, in some cases, the nuclei bulge into the lumina. A lining of very long, compact cilia is present.

Although many empty intertubular spaces remain, there is a definite increase in the number of blood-forming cells and lymphocytes in such spaces. Mitotic figures can be seen in many of the red blood cells. The predominant type of cell in the spaces has a large centrally or eccentrically placed, granular nucleus with a distinct nucleolus, and a heavily granular cytoplasm. These cells are most numerous in intertubular areas adjacent to the coelomic cavity and the dorsal body wall.

Pigment increase is evident particularly in the mesenchymal tissue in the body wall adjacent to the pronephros where pigment accumulations continue to be isolated but of larger mass, and in the distal areas of the nephrostomal cells closely associated with the nuclei. Pigment granules are sparse in the pronephric tubule cells, and are absent from the sinuses. The glomus, which has increased in size, shows pigment invasion varying from small to moderate degree.

The inner region of the pronephric capsule remains thin, but a definite layer of connective tissue is evident. In some specimens, there was slight and in others marked increase in mesenchymal cells adjacent to the outer area of the pronephric capsule. As in the previous stage, there is some growth of connective tissue between the dorsal aorta and the pronephros, but anteriorly only. Moreover, anteriorly, connective tissue seems to be developing in the region of the digestive tract and the dorsal body wall, and extending toward the pronephros.

A few very fine connective tissue cells are enmeshed among the intertubular cells. There was evidence of thin, parallel fibers in the wider band of mesenchymal cells in one specimen.

Expansion of lungs continues. However, only in the most anterior region does the developing lung approach closely to the pronephros; a large coelomic cavity area intervenes posteriorly.

The Himes-Moriber triple stain shows staining characteristics similar to those described in the previous stage. Vacuoles in the cytoplasm, described in the preceding stage, contain material which reacts positively to the polysaccharide stain, and some of the cell inclusions give the same reaction.

4. *Stage of maximum size and beginnings of non-function*

Tadpoles at this stage measured: body length, 10.9-11.9 mm.; tail length, 18.8-20.2 mm.; hind limb length, 1.5-2.2 mm.; maximum width, 6.6-7.2 mm. Specimens in this group exhibited the largest pronephroi, and the pronephroi gave evidence of a highly functional state. There were variations in the shape of the pronephros, not only between specimens, but in the right and left sides of the same specimen. Several pronephroi were of a loose texture, and were rounded in section (Figure 9), but gradually assumed a more elliptical form centrally and posteriorly, while others were more compact and were slightly elliptical in section throughout.

Pronephric tubule sections are highly irregular in shape with extremely large lumina. A very prominent brush border, frequently masked with coagulated material, lines the lumina; the latter also contain clumps and strands of material in small to moderate amounts. In general, there seems to be less difference in epithelial height between the pronephric tubule cells and the collecting tubule cells than in the previous stage. Cells with high cuboidal epithelium show definite cell membranes and very large, central, spherical nuclei with one to four nucleoli of varying sizes. The greater number of pronephric tubule cells are low cuboidal with less definite cell membranes and a central to basal, more oval nucleus. Vacuolated cells, which contain inclusions reacting positively to the polysaccharide stain, are present.

The height of the epithelial cells of the collecting tubules differs little from that of the pronephric tubules, but collecting tubule cells are distinguishable by their more even lumen border. Cell boundaries are indistinct. The nuclei, which are spherical or, more often, oval,

are smaller than those in the pronephric tubule cells, but almost fill these low cuboidal cells. There are fewer inclusions in these cells, and the nuclei stain less intensely than those of the pronephric tubule cells in both the Mallory's and Himes-Moriber stains.

Except for a great increase of pigment in separate granules or clumps, particularly above the nuclei, the nephrostomes appear as previously described. In one specimen, the lower border of the cuboidal cells was beginning to fray, which may indicate the onset of degeneration in this area.

In those pronephroi of loose, round construction, a slight increase of red blood cells and lymphocytes was noted, but some empty sinus spaces remained. However, intertubular areas of oval, compact pronephroi of this stage were rapidly being filled with red blood cells showing mitotic divisions and granulocytes of many types.

An increase of pigment is seen in both the outer band of mesenchymal tissue, and, for the first time, in the cells of the pronephric capsule adjacent to the coelom. Collecting tubule cells also show a marked increase of pigment, particularly in the region of the cell above the nucleus. Scattered granules are also evident in pronephric tubule cells, and small pigment masses are present among the inclusions of vacuolated cells. Intertubular areas of the pronephroi are still free of pigment. A great increase of pigment is seen in some glomi, while others are very similar to the previous stages.

Continued connective tissue development in the region of the dorsal aorta, digestive tract, and advancing lung was noted in all specimens. This increase is greatest anteriorly, but extends more posteriorly than in previous stages. In some specimens, the developing connective tissue formed a sheet in the most anterior region, obliterating communication of the pronephros with the coelomic cavity. In some pronephroi, mesenchymal cells and fine strands of connective tissue were interspersed in the intertubular spaces.

Rapid growth of the lung continues. Anteriorly, it is generally in very close proximity to the pronephros, there being only a very small space between the pronephric capsule and the enveloping connective tissue of the lung bud. In one specimen, these two tissues were seen to meet for a short distance.

There is an increase of polysaccharide-positive material along the brush border and in vacuolated cells, and polysaccharide-positive and protein-positive materials are present within the lumina (Figure 8).

The slightly different staining characteristics of nuclei of the pronephric and collecting tubule cells described in the previous stages were also observed here.

5. *Stage of progressive signs of degeneration*

Tadpoles of this stage displayed the following measurements: body length, 11.0-13.2 mm.; tail length, 20.5-24.2 mm.; hind limb length, 3.0-6.7 mm.; maximum width, 7.0-8.2 mm. More marked signs of pronephric degeneration become apparent during this stage. The following are considered to be characteristics of the degenerative process: a reduction in size of the organ, fraying and disruption of basal and lumen borders of tubule cells, an increase of pigment, an increase of vacuolated cells in the pronephric tubules containing pigment or other inclusions, an increase of secretory or waste materials in the lumina, the collapse of tubules, an increase of blood cells and lymphocytes in the intertubular spaces, the invasion of the intertubular spaces by connective tissue fibers and cells, distortion of nuclei, and the increase and differentiation of connective tissue in adjacent tissues and organs. The closing off of the pronephric chamber by increased lung development also occurs during this time.

The phenomena about to be described for this stage occurred in varying degrees in the seven specimens of this group which were sectioned (Figures 10, 11, and 12), and variations in degenerative progress were observed even between right and left pronephroi of the same specimen. Only two of the specimens showed a slight decrease in pronephric size, but a marked decrease in size was apparent in the other five specimens. Anteriorly, the organ is either triangular or elliptical in section, centrally, it is elliptical, and posteriorly, it is elongated elliptical.

Epithelium of the pronephric tubules is high cuboidal. The pronephric tubules have smaller lumina than in the previous stages (Figures 10 and 11). Material is present within the lumina, and the increased secretion or accumulation of waste products causes the brush border to appear as definite peaks or to assume a very irregular foam-like condition (Figure 3). Fraying and disruption of the upper border was noted in some instances, while dilation and collapse of tubules was apparent in fewer specimens. Many vacuolated cells are present which contain clumps of material (Figure 8). In some cells, the nuclei are oval and more basal, but generally, the cellular inclu-

sions and nuclear appearance are very similar to those previously described.

Collecting tubules have a low cuboidal epithelium and a large lumen which is frequently devoid of material, particularly in the anterior region. The tubules do not appear to be in the stretched condition described in previous stages, and both an intact basal border and a smooth lumen border are evident. Moreover, the nuclei, smaller than pronephric tubule cell nuclei, are oval or spherical, are centrally located, and do not bulge into the lumen as they do in the previous stage.

Except for the fraying of the border and the presence of some material within the lumen in one specimen in this group, there is no obvious change in the segmental duct. The elongated, expanded sections of the segmental duct are intact and in good condition while sections of pronephric tubules in the same vicinity appear to be undergoing degeneration.

Nephrostomes showed little change except a breaking away of the basal border in some cases and greatly increased amounts of pigment which almost obscure the cytoplasmic contents of the nephrostomal cells. Large, prominent nuclei remain.

A tremendous increase in intertubular cells was observed in all specimens (Figure 10). All available sinus spaces are packed with red blood cells and even greater numbers of lymphocytes; division figures in erythrocytes are numerous.

In addition to an increase of pigment in the form of separate granules and small clumps in the nephrostome and pronephric tubule cells, and as fine granules in the collecting tubule cells, pigment is now found in the intertubular sinus areas (Figures 11 and 12). Masses seem to be migrating from the dorsal body wall to the connective tissue along the inner area of the pronephros. The connective tissue around the dorsal aorta contains a large pigment accumulation while some glomi show an increase in pigment over previous stages. There seems to be little change in the outer band of mesenchyme.

Development of the connective tissue in the structures and organs adjacent to the pronephros is pronounced. In the anterior region of the pronephric area, the connective tissue extends from the region of the dorsal aorta, and becomes attached to the pronephros, lessening the communication of the nephrostomes with the coelomic cavity (Figure 12). This attachment continues through many sections. From the regions of the digestive tract, developing lungs, and the dorsal

body wall, connective tissue growth converges toward the pronephros (Figure 11). Within the pronephros, a framework of fibers seems to be advancing from the exterior to the interior. It is noteworthy that in the two specimens which showed less regressed pronephroi, connective tissue development was not nearly as great as in the specimens which showed signs of degeneration. Degenerating tubules seem to be encircled by a network of connective tissue fibers (Figure 13).

Rapid expansion of the lungs continues. Anteriorly, the connective tissue around the advancing lung has made contact with the pronephric capsule (Figure 11). This condition usually prevails only in the anterior region, and a coelomic space between the lung and the pronephros is present in the central and posterior regions. However, the distance between the lung and the pronephros is markedly narrower than in previous stages.

Pronephric tubule cells have large accumulations of material reacting positively to the polysaccharide stain, particularly in the region of the cells nearest the lumen, and clumps of similarly staining, coagulated material or foam-like masses were observed along the lumen border. The lumina of collapsed tubules are filled with polysaccharide-positive material, but tubules which are more intact often contain strands of material which is protein-positive.

6. *Advanced stage of degeneration*

Measurements of the tadpoles at this stage are: body length, 11.1-13.0 mm.; tail length, 23.8-28.4 mm.; hind limb length, 9.6-12.6 mm.; maximum width, 6.4-8.1 mm. At this stage, the tail length is maximal, and the fore limbs are well developed, but have not broken through. The pronephros of one specimen only was similar in size and appearance to that of the preceding stage. The pronephroi of the other specimens became narrow, shrunken, and rectangular (Figure 14). Usually, in cross section, there is a tapering toward the anterior and posterior ends of the structure, and there is a slightly elliptical area toward the center in which are located the remaining tubules. The degenerative process seems to take place anteriorly first, as very little of the pronephros remains in that area. Progressively more intact tubules were observed in the central and posterior regions.

Anterior pronephric tubules have lumina collapsed or filled with coagulated material. Membranes between adjacent cells are no

longer visible, and fraying or disruption of the free surface and basal membranes is present. Posteriorly, the pronephros is larger, and there are more tubule sections present than in the degenerated anterior region. Some of the tubules are intact, and contain nuclei and cytoplasmic constituents similar to those previously described. Other tubules are dilated or collapsed. A continued increase of heavily pigmented, vacuolated cells was noted. Pigment has permeated some of the nuclei, and in some instances, a zone, clear of pigment, was seen surrounding intact nuclei. With each succeeding stage, there is a general increase of pigment within the tubule cells, in the connective tissue surrounding the pronephros, and in structures and organs adjacent to it. However, the presence of pigment within the nuclei was not previously observed.

Collecting tubule cells have also degenerated in the anterior region, but in the posterior region, they seem to have undergone less degeneration than the pronephric tubule cells. Basal and lumen borders are usually intact. These tubules stain less intensely than pronephric tubules. Sinus areas still contain erythrocytes, some of which seem to be degenerating, and masses of lymphocytes. Except for a slight decrease in size, the glomus remains intact (Figure 14) and hangs freely in the pronephric chamber.

Observations of serial sections from anterior to posterior show that the layer of connective tissue connecting the dorsal aorta, the digestive tract, and the developing lung with the pronephros extends considerably farther posteriorly than in the previous stage. Even in the posterior regions, where there is less connective tissue developed, connective tissue from the area of the dorsal aorta greatly limits the size of the pronephric chamber; there is very little space surrounding the glomus. The whole pronephros is permeated with connective tissue strands. The wide, outer area of mesenchymal cells is now replaced by differentiated muscle fibers and cartilage (Figure 14).

7. *Stage of completed degeneration*

Measurements for the tadpoles of this stage were: body length, 11.3-12.4 mm.; tail length, 20.9-23.1 mm.; hind limb length, 14.2-14.4 mm.; fore limb length, 6.0-7.9 mm.; maximum width, 4.9-6.2 mm. This is the stage of the beginning of tail resorption and the first protrusion of forelimbs. In all the specimens, a further shrinkage of the pro-

nephros was apparent. Anteriorly, the remains of the pronephros are confined to a small area, rectangular in section; centrally, it is slightly elliptical, tapering to a narrow strand of tissue on both sides (Figure 15); posteriorly, it is long, narrow, and rectangular.

Both pronephric and collecting tubules are in a degenerated condition. Anteriorly, remnants of only a few tubule sections are visible, and these are completely collapsed and knot-like in appearance (Figure 13). Cross sections of central and posterior regions show an increase of remaining tubule sections over the anterior regions, but all are collapsed or have lumina completely filled with dense, coagulated material and pigment. The cytoplasm of the tubule cells has shrunken so that there are large, clear areas around the tubule sections. Neither basal, striated, nor brush border was detectable, and numerous vacuolated cells containing coagulated material and pigment were observed. Of the cellular components, nuclei appear to be the last to undergo degeneration as many basal, spherical nuclei with distinct nucleoli were still observable in the remaining cells.

At this stage, the segmental duct has the same aspects of degeneration as the pronephric and collecting tubules, and appears to be no longer functional. It, too, has a large, clear area surrounding the very small clump of degenerated remnants, and the sinus in which it is embedded is filled with erythrocytes and lymphocytes.

Although the cilia are still present, no cell structure could be distinguished in the nephrostome. The basal border is disrupted, and masses of pigment obscure any remaining cellular components. In some regions, the lumen of the nephrostome is filled with lymphocytes.

There appears to be a decrease of intertubular cells in the remaining sinus areas. These cells are interspersed among the network of connective tissue fiber cells; some of the erythrocytes appear to be distorted. This decrease and disorganization of cells was apparent in the glomus which has become smaller (Figure 15), and stains less intensely with Mallory's stain than in previous stages.

The cytoplasmic contents of the tubule cells seem to have been replaced by pigment. Pigment also accompanies the migrating and rapidly developing connective tissue stroma which is contained within and also surrounds the pronephros. The glomus also contains massive clumps and smaller scattered pigment accumulations. Even blood cells contain pigment granules, a condition which may be responsible

for their distortion mentioned previously. However, recently differentiated muscle and cartilage in the vicinity are free of pigment.

Within the pronephros, the connective tissue fibers have formed a well-defined network of bands which surround the clear areas and the no longer functioning tubules (Figure 13). Connective tissue strands from around the dorsal aorta join fibers which have extended from the dorsal body wall and attach themselves to the pronephros along the coelomic cavity area. This development encloses the glomus within a very small chamber which is no longer in communication with the remainder of the coelomic cavity. Differentiation of mesenchymal tissue into muscle and cartilaginous tissue in the surrounding regions has progressed rapidly. A wide band of longitudinal muscle fibers originating in the area of the dorsal aorta passes along the outer area of the pronephros, and extends beyond the posterior confines of the structure (Figure 15). In some instances, this band split longitudinally and resulted in the penetration of sections of the muscle band into the pronephros itself. Circular muscles and a wide band of cartilage occupy the area between the longitudinal muscle band and the integument. Anteriorly, the connective tissue surrounding the lung is attached to the tissue layer developed along the coelomic cavity border of the pronephros. Although in the central and posterior regions there is less connective tissue around the lung, the rapid increase in lung development has brought this organ into very close proximity to the pronephros.

Connective tissue fibers stained intensely polysaccharide-positive in Himes-Moriber stain. The coagulated material in the lumina of the remaining tubules stained similarly. Small spherical polysaccharide-positive and larger protein-positive cellular inclusions were still observable within the degenerating tubule cells.

8. *Completed metamorphosis*

Measurements of animals in this group were: body length, 11.3-12.7 mm.; tail length, 1.0-14.0 mm.; hind limb length, 14.6-16.0 mm.; fore limb length, 6.8-8.4 mm.; maximum width, 5.0-6.2 mm. There is little difference in hind limb length from the preceding stage, but rapid resorption of the tail and significant fore-limb development takes place. The transition from the aquatic to the terrestrial environment was made by some of these specimens.

As metamorphosis continues, the pronephric area continually de-

creases in size, and now occupies a very small fraction of the original area. Anteriorly, a small rectangular mass of connective tissue fibers and blood cells represents the degenerated pronephros. Centrally and posteriorly, the pronephric remnants appear as a more compact, rectangular mass or is slightly elliptical, tapering to a thin strand of tissue at both sides (Figure 16).

Anteriorly, there are no remains of tubules. Within the loose arrangement of connective tissue fibers are clumps of red blood cells, lymphocytes, pigment, and a concentrated bundle of dense connective tissue fibers. Toward the center, and continuing posteriorly, some of the specimens had tubule remnants similar to those described for the preceding stage. However, other specimens appeared to have undergone further degeneration. In these, there was no vidence of tubules as such. Clear areas, encircled by connective tissue, represent former tubule positions (Figure 16). In the center of the clear area, one or more of the following are found: large globules of coagulated material, completely disorganized cells, scattered or isolated extruded cytoplasmic inclusions, distorted nuclei, and pigment. Except for its location, the remnant of the segmental duct is indistinguishable from other tubule remnants. In some instances, the remnants and enveloping sinus did not appear to be drawn toward the dorsal aorta as was noted in previous stages.

There are no signs of the nephrostomes except in the posterior regions. Although the structure has been closed off from the coelom, there remain tufts of cilia bounded by degenerated cells.

Anteriorly, a marked decrease of lymphocytes is evident in the pronephric area; some specimens, however, had concentrated masses of red blood cells. In the more posterior regions, large numbers of various cell types are enmeshed in the connective tissue stroma. Some of these cells are distorted and pigmented. Although the amount of pigment varies from one specimen to another, in general, tubular remains, intertubular spaces, connective tissue enveloping the pronephric area, the glomus, and adjacent tissues have a large pigment accumulation. However, the most notable single increase of pigment is along the connective tissue bands which have penetrated the pronephros, and surrounded the collapsed and degenerated tubules (Figure 16).

Great variation in size and appearance of glomi was noted among specimens of this group. Some were still large, stained intensely, and contained many blood cells; others were very small and shrunken,

stained significantly less intensely than in previous stages, and were almost depleted of cells. The investing peritoneal layer is still sharply delineated, and stains polysaccharide-positive with the Himes-Moriber stain. In all specimens, the glomi become wedged between connective tissue which extends from the region of the dorsal aorta to the pronephros and the expanded lung. This is the result of rapid growth, development, and metamorphic changes. The glomerular cavity is, therefore, narrow, and the glomus touches adjacent tissue on both sides. In areas where the lung has expanded and fused with the newly formed Mullerian duct, sections of the glomerular cavity appear to be reduced to a small rectangular space cut off from the remainder of the coelomic cavity (Figure 17).

Beginning anteriorly and extending through many sections, connective tissue fibers interconnect the dorsal aorta, digestive tract, lung, dorsal body wall, and the pronephric area. This completely obliterates the coelomic cavity in this region. The connective tissue fibers within the pronephros, at these levels, are loosely arranged and somewhat parallel. Some specimens, however, had a mass of compact fibers embedded within the pronephros (Figure 16). Where tubule remnants were present in the more posterior regions, connective tissue fibers formed a dense network, even penetrating cellular remains in some cases. Muscle and cartilage development in the region of the outer body wall continues, and much of the mesenchymal tissue around the dorsal aorta is differentiating into muscle tissue.

In serial sections, the position of the lung in relation to the pronephros is as follows: in the most anterior sections, the complete, widely expanded, flattened edge of the lung is attached to the pronephros. As has been previously noted, the tissue around the lung, with adjacent tissues, form an uninterrupted layer over the coelomic cavity. More centrally, there is less connective tissue around the lung, and only the tip of the lung is attached to the peritoneal layer bordering the pronephros. This results in the formation of two small cavities on either side of the expanded lung. More posteriorly, the lung does not touch the pronephric area, and the coelomic cavity appears larger here. However, this enlargement does not continue. The glomus wedged between the dorsal aorta region and the lung, the expansion of blood vessels associated with the lung, the fusion of the Mullerian duct with the lung (Figure 17), and the rapid growth of the liver, all tend to reduce the coelomic cavity area. Even more

posteriorly, the lung tapers in size, and the liver occupies an increasingly larger area.

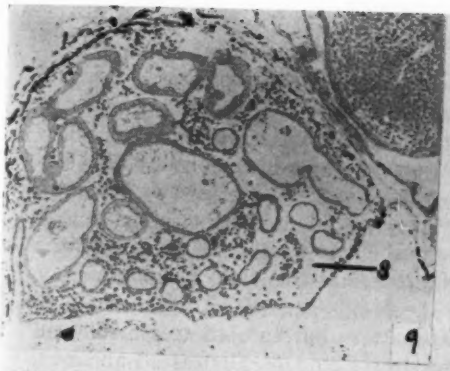
B. *Pronephric Regeneration in Thiourea-Treated Tadpoles*

To study the possible relation of thyroid function with pronephric degeneration, specimens were treated with 0.03% thiourea to inhibit thyroid activity. Treatment was initiated from the hind limb bud stage to the 2.0 mm. hind limb stage, and continued for approximately three and one-half months after the completion of metamorphosis of the control animals. This concentration and stage of initiation of treatment results in continued rapid growth of the specimens while it completely inhibits tail resorption and fore limb protrusion, and partially inhibits hind limb growth. Giant tadpoles develop with body length, tail length, and width far exceeding those of the largest of the control specimens. However, after the specimens are approximately two and one-half months old, no further increase in size occurs. During treatment, the skin becomes very tough, a wide, extremely muscular, curved tail develops, and the broad, rounded tadpole head and body form are retained. Although in all cases hind limb development is partially inhibited, some hind limb growth does occur in all specimens. None, however, approaches the minimal hind limb length of metamorphosed controls. Moreover, the development is extremely slow as compared with controls. Great variation in hind limb growth of treated animals was noted in the 1956 groups. In some specimens treated over a long period of time hind limb growth did not exceed 2.0 mm. while other specimens developed much longer hind limbs. The individual variation is greater the older the specimens are at the time of the beginning of treatment. Very little variation was noted among the 1957 specimens, and although they were maintained for a longer period than the 1956 specimens, none attained hind limb growth greater than 5.0 mm. Treatment was initiated for all the 1957 specimens at the hind limb bud stage, and this probably accounts for the greater uniformity observed.

Detailed comparison was made of the pronephroi of thiourea-treated and control tadpoles fixed at various intervals after the beginning of treatment. It was found that the changes in the pronephros, like those in the hind limb, varied considerably in the 1956 groups, but showed little variation in the 1957 groups where all the animals were at precisely the same stage when treatment was begun.

PLATE II

9. Anterior section through the pronephros of a control specimen at stage 3. X 50.
10. Central section through the pronephros of a control specimen at stage 4. X 50.
11. Central section through the pronephros of a control specimen at stage 4. Note growth of connective tissue from the lung which is attached to the pronephric capsule. X 50.
12. Anterior section through the pronephros of a control specimen at stage 4. X 50.
13. Section showing degenerating tubule section encircled by connective tissue fibers. X 475.
14. Central section through the pronephros of a control specimen at stage 5. X 50.
15. Central section through the pronephros of a control specimen at stage 6. X 50.
16. Central section through the pronephros of a control specimen at stage 7. X 50.
17. Section showing newly developed Mullerian duct at the site of the degenerated pronephros of a control specimen. X 50.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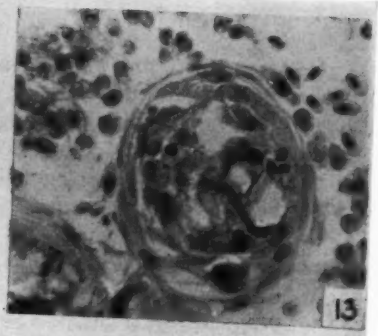
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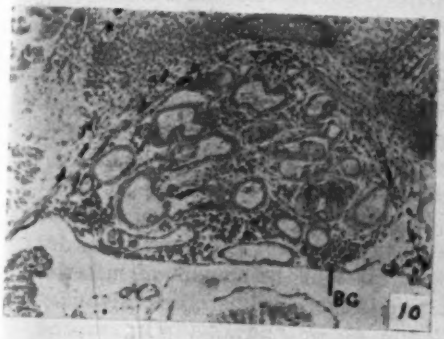
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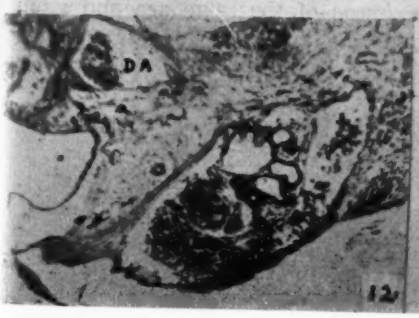
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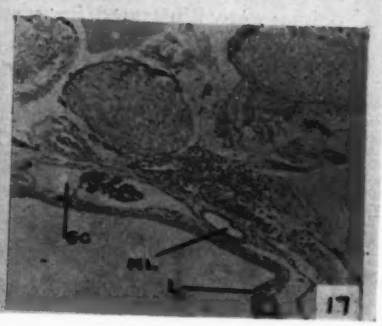
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Both control and experimental animals fixed after five to seven days have large, fully functional pronephroi which do not differ significantly in their histology (Figures 18 and 19). However, the size of the pronephros is greater in the treated animals (Compare Figures 18 and 9, 19 and 7), a fact which is worthy of note since the two groups do not show any differences at this time in body size or in hind limb length.

Specimens sectioned 8-13 days after the start of treatment still showed differences in pronephric size (Compare Figures 20 and 21), but controls now have more coagulated material along the brush borders and within the lumina of the tubules than do the experimentals. Controls also have more intertubular lymphocytes and erythrocytes and more pigment and connective tissue in the pronephric area. It may also be noted that the pronephric chamber is now more restricted in controls due to increased size of the liver, intestines, and lungs.

More obvious differences in histological structure appear in specimens treated from 13-20 days. Controls now have more compact pronephroi than previously, and many tubules are undergoing degenerative changes as shown by increases in the amount of material in the lumina, invasion of connective tissue, and more extensive pigmentation (Compare Figures 12 and 22, 23 and 24). Organs and tissues adjacent to the pronephros have increased in size, and the organ is thus compressed. Pronephroi of treated tadpoles, however, retain the characteristics of the fully functional organ (Figures 23 and 24). However, some changes which must be regarded as forerunners of degeneration are evident. The pronephros has become somewhat more compact, growth of connective tissue has increased, and the openings of the anterior nephrostomes have become restricted.

After 21-30 days, control animals are all in advanced stages of pronephric degeneration (Figure 25). The organ in the experimentals still exhibits signs of functional activity but varying degrees of degenerative change are now discernible (Figure 26). It appears that extensive alterations in the vascular pattern in the pronephric area occur at this time, and may well be a major factor in influencing the condition of the pronephros. If blood vessels are adjacent to or around the pronephric tissue, signs of degeneration are fewer. The amount of connective tissue present also seems to be a factor. These points were illustrated in two specimens of the same age and size treated for 24 days (Compare Figures 27 and 28).

Control tadpoles metamorphosed 30-35 days after the beginning of the experimental period and, of course, showed completely degenerated pronephroi (Figure 16). Experimental animals still show a large pronephric area at this time (Compare Figures 16 and 29), but continuing degeneration is clearly evidenced.

Some treated specimens were maintained as long as three and one-half months after the metamorphosis of controls. During this time further degeneration of the pronephros occurred but apparently at a slower rate, and, even though the tubules were ultimately almost completely disintegrated and intertubular spaces became filled with cells and thick connective tissue fibers, the area of the pronephros remained quite large. Thus the pronephros still contrasts strongly with that of the metamorphosed control (Figures 30-34). Growth of intestine, liver, and lungs has continued, and the amount of connective tissue present far exceeds that seen in completely metamorphosed controls. As a result the communication of the nephrostomes with the body cavity has been completely cut off in most specimens (Figures 30, 31, 33, and 34).

The variability of response in the experimental series of 1956 is of some interest for it shows that the exact stage at which treatment with thiourea is initiated is most important in determining the results. In the 1956 series, the stage of development varied from limb bud to 2.0 mm. Although this difference is relatively slight, it was found that, after as little as 14 days treatment, the pronephroi of experimental specimens showed a variation from completely functional organs of maximal size to pronouncedly degenerated kidneys. This variation continued to be apparent at all later ages. When contrasted with the lack of variation found in the treated animals of the 1957 series, this indicates clearly that slight differences in the stage of development of the tadpole are accompanied by well-marked differences in the effectiveness of thiourea treatment in inhibiting metamorphic processes.

C. *Pronephric Degeneration in Thyroxin-Treated Tadpoles*

Specimens in which treatment was begun at the limb bud stage survived an average of six days. By this time, the body length shows a decrease of 3.0 mm., the hind limbs reach a length of 4.0 mm., and have differentiated digits, one or both fore limbs appear, and the tail length is decreased from 20.0 mm. to 3.0 mm. Specimens treated for

PLATE III

18. Anterior section through the pronephros of a specimen placed in 0.03% thiourea solution at the hind limb bud stage and fixed 6 days later. X 50.
19. Anterior section through the pronephros of a specimen placed in 0.03% thiourea solution at the hind limb bud stage and fixed 7 days later. X 50.
20. Central section through the pronephros of a specimen placed in 0.03% thiourea solution at the hind limb bud stage and fixed 11 days later. X 50.
21. Central section through the pronephros of a control specimen corresponding in hind limb length and age to the thiourea-treated specimen of Figure 20. X 50.
22. Anterior section through the pronephros of a specimen placed in 0.03% thiourea solution at the hind limb bud stage and fixed 16 days later. X 50.
23. Central section through the pronephros of a specimen placed in 0.03% thiourea solution at the hind limb bud stage and fixed 16 days later. X 50.
24. Central section through the pronephros of a control specimen of the same age as the thiourea-treated specimen of Figure 23. X 50.

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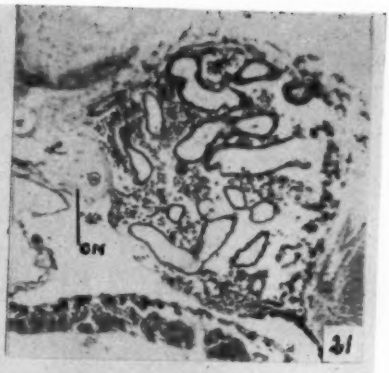
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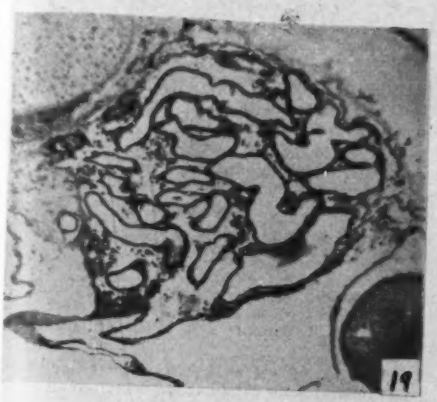
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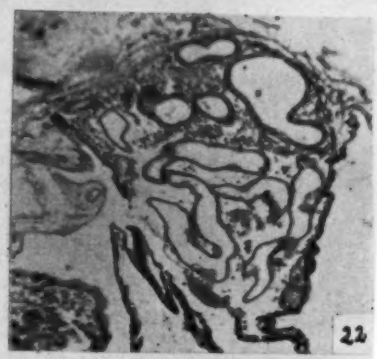
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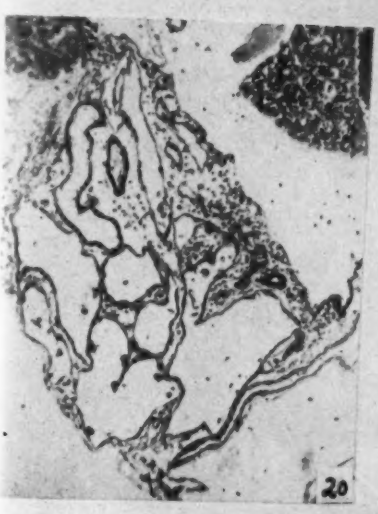
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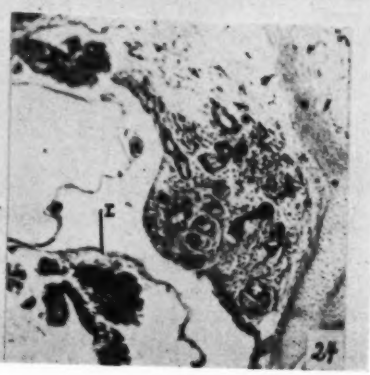
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PLATE IV

25. Central section through the pronephros of a control specimen of the same age as the thiourea-treated specimen of Figure 26. X 50.

26. Central section through the pronephros of a specimen placed in 0.03% thiourea solution at the hind limb bud stage and fixed 22 days later. X 50.

27. Central section through the pronephros of a specimen of a 1956 series placed in 0.03% thiourea solution and fixed 24 days later. Note connective tissue and lung growth closing off nephrostomal communication with the coelomic cavity. X 50.

28. Central section through the pronephros of a specimen of a 1956 series placed in 0.03% thiourea solution and fixed 24 days later. Note unobstructed nephrostome and greater coelomic cavity area as compared with specimen of the same age and length of treatment, Figure 27. X 50.

29. Central section through the pronephros of a specimen of a 1957 series placed in 0.03% thiourea solution and fixed 31 days later. Corresponding controls have degenerated.

30. Central section through the pronephros of a specimen of a 1956 series placed in 0.03% thiourea solution and fixed 48 days later. Note the large blood vessels in close proximity to the remaining tubule sections. Note compression of the tubules by adjacent tissue and organ growth. X 50.

31. Anterior section through the pronephros of a specimen of a 1957 series placed in 0.03% thiourea solution and fixed 52 days later. Note complete degeneration of tubules, but large pronephric area is maintained. X 50.

32. Central section through the pronephros of a specimen of a 1956 series placed in 0.03% thiourea solution and fixed 52 days later. X 50.

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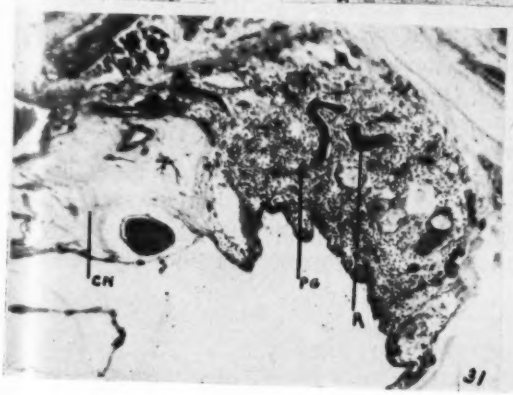

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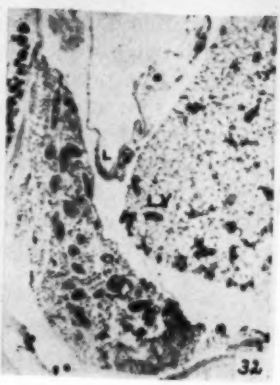

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twenty-four hours already show a greater compactness of the pronephros than do controls (Compare Figures 35 and 9). After two days, this is still more evident, and an extensive growth of connective tissue unites the pronephroi with the lungs and digestive tract. The pronephric tubules still appear to be in functional condition, however. By the third day of treatment (Figure 36) a thick capsule has formed about the organ, intertubular cells are abundant and some tubules seem to be compressed by the surrounding capsule or by the adjacent developing tissues. The lung is attached to the pronephros throughout a large area. On the fourth day of treatment, much degeneration of pronephric tissue is evident, and by the fifth day (Figure 37) degeneration is almost complete, since specimens preserved on the sixth day show little further change.

When the thyroxin treatment was initiated at the 1.5-3.0 mm. hind limb stage, the external changes occurred more rapidly than in younger animals and the specimens succumbed after 3-4 days of treatment. The decrease in body length of these tadpoles is very slight, but the hind limbs grow 5.0-6.0 mm., and show complete differentiation of digits and joints. Both fore limbs always appear, and the tail length changes from 21.0 mm. to 7.0 mm. Changes in the pronephroi, like those in the external features, occur more rapidly than in the tadpoles treated at the hind limb bud stage. After only 24 hours, the pronephroi are greatly reduced in size, and seem to be compressed between other tissues (Figure 38). Many inclusions and vacuoles are present in the tubules. The lung, however, is still widely separated from the pronephros. In animals treated for two days (Figure 39), the tubules are small and usually collapsed, a great increase in intertubular cells has occurred, and a thick connective tissue capsule is present. By the third day (Figure 40), all of these changes have progressed further, and the growth of surrounding organs has partially cut off communication of the nephrostomes with the coelomic cavity.

It is noteworthy that, although the sequence of changes in degeneration of the pronephros in the thyroxin-treated tadpoles corresponds to that seen in the normal course of metamorphosis, several seemingly important features of the normal process did not occur. 1) Invasion of the pronephros by large amounts of pigment was not as generally seen in the accelerated specimens. 2) There was no indication of any marked decrease in the blood supply to the pronephros during its degeneration in the thyroxin-treated animals. In both control and

thiourea-treated specimens, the presence of large blood vessels in the vicinity of the pronephros always seemed to be accompanied by persistence of functional tubules. Such vessels were abundant in the thyroxin-treated animals, but did not prevent degeneration. 3) Communication of the nephrostomes with the coelomic cavity was usually unimpaired in the thyroxin-treated tadpoles. The blocking off of nephrostomes by the growth of surrounding tissue always seemed to precede degeneration of tubules in control and thiourea-treated animals. Most of the thyroxin-treated specimens in which advanced pronephric degeneration had occurred showed no such blockage (Figure 41).

Discussion

In this study, it was found that normal degeneration of the pronephros of *Rana sylvatica* starts at about the same time as the beginning of rapid growth of the hind limbs, and that the pronephros loses the histological characteristics of a functioning organ before tail resorption begins. Pronephric degeneration is thus an early feature in the metamorphic pattern. The histological features of the degenerative process have not previously been studied in detail, but the brief observations made by Field (1891), Shore (1901), Fraser (1950), Jaffee (1954), and Koszarek (1956) have been, for the most part, confirmed here. Jaffee (1954) reported that in *R. pipiens* no Mullerian duct forms at the site of the degenerated pronephros. In *R. sylvatica,* however, the Mullerian duct does form at the level of the completely degenerated pronephros (Hall, 1904), as was confirmed in this study. The degenerative changes always appear first at the anterior end of the organ and then proceed caudally. The first kidney elements to exhibit such changes are the nephrostomes. The changes are then seen successively in the pronephric tubules, collecting tubules, and the segmental duct. Notable features of the degenerative process in the cells are: fraying and disruption of the basal and lumen borders of the tubule cells; an increase of pigment granules, at first above the nucleus and later scattered throughout; an increase of vacuoles containing pigment or other inclusions; and finally, the collapse of cells and distortion of nuclei. With respect to the entire organ, degeneration also involves compression and decrease in size, progressive increases in lymphocytes and erythrocytes in the sinuses, invasion and encapsulation of the pronephros by connective tissue, a filling up of lumina of tubules with secretory or waste materials, and an extensive

PLATE V

33. Anterior section through the pronephros of a specimen of a 1956 series placed in 0.03% thiourea solution and fixed 41 days later. X 50.

34. Anterior section through the pronephros of a specimen of a 1957 series placed in 0.03% thiourea solution and fixed 47 days later. X 50.

35. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at the hind limb bud stage and fixed 24 hours later. X 50.

36. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at the hind limb bud stage and fixed 3 days later. X 50.

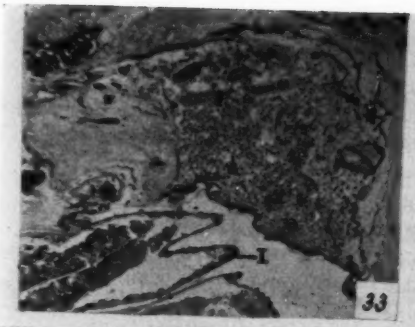
37. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at the hind limb bud stage and fixed 5 days later. X 50.

38. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at approximately the 2 mm. hind limb stage and fixed 24 hours later. X 50.

39. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at approximately the 2 mm. hind limb stage and fixed 2 days later. X 50.

40. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at approximately the 2 mm. hind limb stage and fixed 3 days later. X 50.

41. Section through the pronephros of a specimen placed in a solution of one part thyroxin to ten million parts water at approximately the 2 mm. hind limb stage and fixed ? days later. Note the advanced degeneration of the pronephros although the nephrostome, glomus, and coelomic cavity are unobstructed by connective tissue and organ growth. X 50.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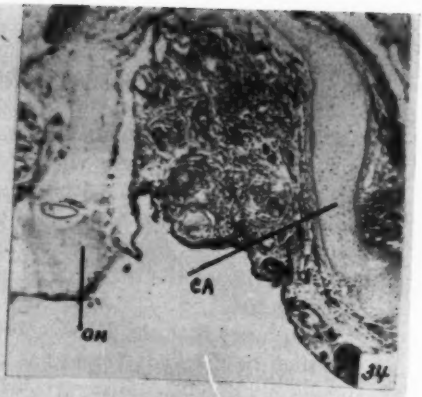
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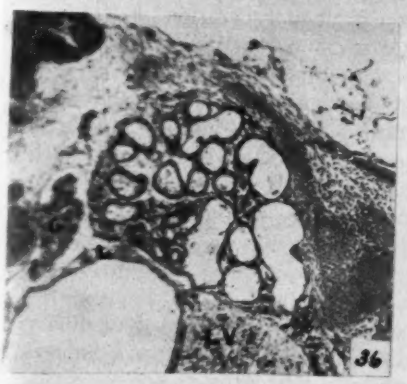
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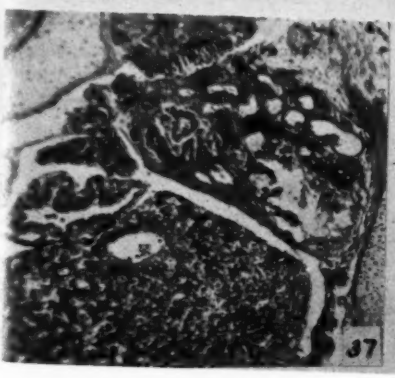
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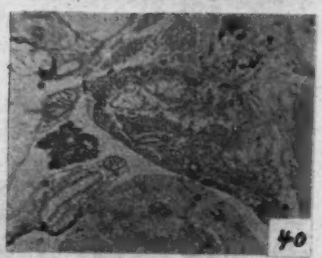
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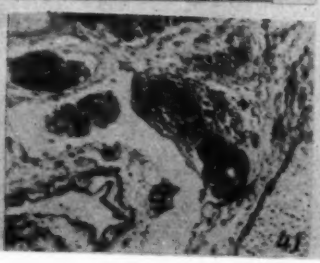
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accumulation of pigment in the organ. The pigment accumulation may be simply a result of the cessation of excretory activity by the pronephros. Lim (1920) has suggested that pigment, produced as a metabolic product, is eliminated by the pronephros in the tadpole. If this is true, the gradual piling up of pigment in the organ, as excretory activity of the pronephros ceases and degeneration occurs, might be expected.

It appears that the DNA content of the collecting tubule cells may be slightly different from that of the pronephric tubule cells. The nuclei stain blue. However, when rapid division and expansion of pronephric tubule cells occurs, the resulting nuclei at first resemble more closely nuclei of collecting tubule cells. The staining method used is not specific for the RNA component of the nucleolus, but nucleoli of collecting tubule cells are smaller and take a less intense protein-positive stain than nucleoli of pronephric tubule cells. Possibly variation in the absorptive and secretive functions of the pronephric tubule cells are responsible for differences in the size and staining properties of their nuclei. The abrupt transition from pronephric tubule cells to collecting tubule cells may also be reflective of their individual functions.

The present observations on pronephric degeneration and simultaneous growth and differentiation of surrounding tissues and organs indicate the importance of secondary factors in the process of pronephric degeneration. This view of the significance of secondary interactions in understanding important features of morphogenesis is stressed by Danforth (1933). General increase of body size toward metamorphic completion, compression of the pronephros due to bone, cartilage, and muscle differentiation anteriorly, posteriorly, and in the region of the integument, changes in the circulatory system; as well as increased growth of lungs, liver, and intestine may be contributing factors in pronephric degeneration.

A number of hypotheses have been offered concerning the primary factors which may be involved in the initiation of degenerative changes in the pronephros. Shore (1901) reported that, after the origin of the mesonephros, the segmental duct becomes narrower and finally completely occluded in the region between the pronephros and the mesonephros. He suggested that it is this closure of the drainage duct which causes the cessation of function and ultimate degeneration of the pronephros. However, as noted by both Jaffee (1954) and Kos-

zarek (1956), pronephric degeneration is first seen anteriorly and proceeds caudally, and advanced degeneration of the anterior pronephric tubules can be found before any changes occur in the segmental duct. Indeed, as mentioned previously, the present results indicate that the segmental duct is the last of the kidney elements to undergo degeneration.

Shore (1901) also emphasized the importance of the intimate relation of the posterior cardinal veins with the pronephros. These veins form vascular networks or sinuses around the tubules. During the development of the tadpole, however, the posterior cardinals decrease in size and importance as the posterior vena cava takes over the function of drainage of the posterior part of the body. It seems possible, therefore, that the decrease in vascularity in the pronephric region might be a primary factor in the degeneration of the head kidney. The present observations show significant changes in the vascularity of the pronephros but, again, these seem to follow rather than precede the beginning of the degenerative changes in tubules and nephrostomes.

Gradual loss of function by the pronephros because of the increasing functional activity of the mesonephros is another possible explanation for the final disappearance of the head-kidney. It is, of course, true that the mesonephros is well developed at a quite early stage and that, as Jaffee (1954) pointed out, there is a period during which both organs share the excretory function. However, the normal degenerative process seems to occur rather suddenly soon after the pronephros has attained maximum size and regardless of the relative size and functional state of the mesonephros. Moreover, both the work of Koszarek (1956) and the present study indicate that with thiourea treatment the mesonephroi become much larger than normal, yet early degeneration of the pronephroi does not result.

Jaffee (1954) reported that degeneration of the pronephros seems to follow rapid expansion of the lungs, and suggested that contact between the two organs may be a factor in the process. In the present study, growth and expansion of the lungs was seen to be a gradual process extending through the whole metamorphic period. The general growth of tissue in this area does seem important. As the lungs develop, connective tissue in the region grows out to fuse with adjacent organs, including the pronephros, first anteriorly, and then more posteriorly. This growth of connective tissue causes a decrease in the size of the

coelomic cavity around the pronephros and appears to wedge the glomus between the lung and the dorsal aorta. Free access of the nephrostomes to the coelomic cavity is gradually restricted. It may be supposed that these changes could seriously interfere with the function of the pronephros.

Gérard and Cordier (1934) and Fraser (1950) have reported that pronephric tubule cells are both absorptive and secretory, and possess athrocytic abilities whereby they can absorb substances and store them as granules or in vacuoles for some time before expulsion. This action is exercised on colloidal particles of all dimensions, and a gradient of permeability exists the length of the tubules. It is greatest in the region of the nephrostome. Moreover, pronephric tubule cells have phagocytic properties. Water is reabsorbed by collecting tubule cells. In the present study, it was shown that as degeneration advances discrete granules and vacuoles containing clumps of materials, usually staining polysaccharide-positive, increase in pronephric tubule cells as does material along the brush border and within the lumina. Such granules and vacuoles are never seen in the collecting tubule cells. In late stages of degeneration, these vacuoles frequently take up the entire cell area. It seems logical to conclude that cutting off supplies of glomus filtrate and coelomic fluid would hinder the proper functioning of the tubules, and result in an accumulation of waste products. Moreover, it is likely that athrocytic and phagocytic activities would be incapacitated. Degeneration of pronephric tubules and the accumulation of material within their lumina area breaks the continuity of the tubule system of the organ and prevents the passage of fluids through the tubules. This eventually effects the disorganization of collecting tubules and the segmental duct.

Since many of the changes which occur during tadpole metamorphosis have been shown to depend upon the direct action of the thyroid hormone, it was logical to suggest that the degenerative changes in the pronephros may be thyroid-controlled. This hypothesis was advanced in connection with early studies on thyroidectomy (Fulton, 1921; Huxley, 1925) and also on the basis of results of a recent investigation of the effects of goiterogenic drugs (Lynn and Peadon, 1955). The experimental procedures used in the present work were designed to test this possibility.

The experiments in which thyroxin was administered to tadpoles at various stages do seem to indicate that degeneration of the pro-

nephros is a feature which is under thyroid control. When thyroxin (1 part: 10 million) is given at any of the stages studied, all metamorphic changes are rapidly initiated. The histological changes in the pronephros are typical of those seen in normal metamorphosis, and it is noteworthy that they occur even when there has been relatively little growth of surrounding organs and connective tissue. In many of the thyroxin-treated specimens, the glomus was found to be in good condition, and the coelomic cavity in the pronephric region was still quite unrestricted, yet the pronephric tubules were undergoing marked degeneration. It is likely that the degenerative changes are directly attributable to the effect of the thyroid hormone upon the pronephros but it seems that connective tissue encapsulation, interruption of blood supply and the like cannot be excluded as secondary causes.

The effectiveness of thiourea in preventing metamorphosis of tadpoles through its thyroid-inhibiting action was first shown by Gordon, Goldsmith and Charipper (1943). Although this drug has been widely used in experiments on amphibians since that time, no study, with the exception of the work of Koszarek (1956), has been made of the course of degeneration of the pronephros in tadpoles whose metamorphosis has been inhibited. Lynn and Peadon (1955) reported that the pronephroi of thiourea-treated embryos of *Eleutherodactylus martinicensis* do not undergo degeneration at the usual time. Since, however, *Eleutherodactylus* is an anuran with a most unusual developmental history characterized by the absence of an aquatic larval stage, this finding is not directly applicable to a true tadpole.

In the present study, tadpoles of *Rana sylvatica* raised from the hind limb bud stage in a solution of 0.03% thiourea exhibited striking inhibition of metamorphic processes and the hind limbs never exceeded a length of 5.0 mm. Detailed study of the course of degeneration of the pronephroi in treated tadpoles showed that degenerative changes were long delayed, functional pronephroi being retained after those of untreated controls had undergone complete degeneration. Nevertheless, even with continued thiourea treatment, definite degenerative changes did occur. Even though hind limb growth ceased when the limbs had reached a length of 4.0 to 5.0 mm., indicating a cessation of metamorphic changes in these structures, the pronephroi later showed histological indications of degeneration, and finally underwent a considerable decrease in size. However, complete degeneration, as seen in fully metamorphosed controls, did not occur in the

experimental tadpoles. Treated tadpoles maintained even up to three and one-half months after metamorphosis of controls was completed still retained tadpole characteristics.

The fact that definite pronephric degeneration, even though long delayed and incomplete, did eventually occur under conditions of thyroid inhibition might be taken to indicate that the thyroid hormone is not involved in this process. However, this does not necessarily follow, and several possible explanations may be offered. Blacher (1928), Allen (1932), Etkin (1930), and Lynn and Wachowski (1951) reported that various features of the metamorphic pattern have different thresholds of sensitivity to thyroid hormone and that the orderly succession of steps in metamorphosis is due to differences in threshold response in different tissues. Etkin (1930) maintained that this pattern is a constant feature of the transformation of the individuals of any species, and that the sequence of events, therefore, is not controlled by the metamorphosis-inducing substance, but is inherent in the tissues. However, it is conceivable that pronephric degeneration is a feature with a very low threshold, initiated by a level of thyroid hormone much lower than that needed for other metamorphic changes. Possibly the fact that pronephric degeneration begins so early in control animals may be taken as an indication of such a low threshold sensitivity. With this hypothesis one might suppose that, in the present experiments, the thyroid level had already risen sufficiently before thiourea treatment was initiated to stimulate the beginning of pronephric degeneration even though no histological indications of degeneration were as yet discernible. Thus, although thyroid inhibition later became effective and prevented the initiation of other metamorphic changes, the degeneration of the pronephros, having already been stimulated, might ultimately be expected to occur. Such an hypothesis does not, however, seem justified in view of the fact that such a long period elapsed after the beginning of thiourea treatment before any histological signs of degeneration could be detected. Moreover, pronephroi of treated specimens give evidence of maximum size and function about one week after treatment is initiated, and pronephroi of experimentals are significantly larger than the controls although the age and body, tail, and limb measurements are equal. Too, the tubule sections of experimentals are particularly large, and appear to be in a state of hyperactivity. It should be remembered also that the hind limbs underwent some increase in length

after the beginning of thiourea-treatment, indicating that they had definitely begun a progressive metamorphic change, and yet ceased their growth and remained almost static once thyroid inhibition became effective. In this case, changes begun before thiourea was administered clearly were interrupted by the effects of the drug, and there seems no reason to suppose that pronephros degeneration, stimulated prior to treatment, would not also be prevented once thiourea administration was started.

Another possible explanation of the results, based also on the concept of a low threshold sensitivity, is that treatment with 0.03% thiourea did not entirely prevent the production of thyroid hormone but permitted instead a very low production rate. If this were the case, and if the sensitivity of the pronephros to thyroid stimulation were extremely low, it could be supposed that degeneration of the pronephros might occur while other metamorphic features, receiving higher thyroxin levels, would be prevented. That this explanation is doubtful is indicated by the fact that preliminary experiments in which somewhat higher concentrations of thiourea (0.05%) were used gave results similar to those with 0.03%.

If it is to be concluded that the degeneration of the pronephros in the thiourea-treated tadpoles cannot be attributed to stimulation by the thyroid hormone prior to treatment or to stimulation by a continuing low-level hormone production throughout the treatment period, some explanation apart from thyroid hormone effects must be sought. In the description of the normal degeneration of the pronephros, the apparent importance of the many growth and developmental changes, as at least secondary factors in pronephric degeneration, was stressed. It must be borne in mind that thiourea, while inhibiting metamorphic changes, does not inhibit growth and differentiation of tissues not under the control of the thyroid. Throughout this investigation evidence showed that the extent to which growth and differentiation of surrounding tissues and organs had advanced is indicative of the extent of pronephric degeneration. In treated specimens, moreover, the size of lung, liver, and intestines greatly exceeds those of control specimens. As noted by Koszarek (1956) and in the present study, the connective tissue growth in and around the pronephroi of experimentals exceeds that in controls of corresponding stages. It would seem that with nephrostomal communication to the coelomic cavity inhibited, the reduction of the size of the coelomic

cavity, the disappearance of necessary blood supply, and events proceeding from these conditions would render normal functioning impossible and consequent accumulation of waste products inevitable. It is conceivable, therefore, that the cutting off of the blood supply, coelomic fluids, and glomus filtrate could ultimately result in cessation of function of the pronephros and in degenerative changes in the tubules.

The degeneration of the pronephros in thiourea-treated tadpoles, then, may be considered as differing in cause and indeed differing somewhat in nature from that in normal metamorphosis. The present experiments indicate that in the normal metamorphic process the degeneration of this organ is under thyroid control. When thyroid inhibition is produced in the young tadpole, this basis for degenerative changes is removed, and no degeneration takes place for a long time. Nevertheless, ultimate interference with the functioning of the organ by growth of surrounding tissues finally results in histological changes which result in degeneration of the organ. In the latter case, however, the pronephros retains a larger size and a more typical pronephric shape than is seen in the degenerated pronephric structure of a metamorphosed control.

Summary

1. Histological and cytological studies of functional and degenerating stages of the pronephroi of tadpoles of the species *Rana sylvatica* were made with the light and phase contrast microscopes. Comparative studies were made of pronephric degeneration in control and treated specimens through a series of stages. Controls and experimentals were also compared using a number of days of treatment as a criterion of comparison.

2. Pronephroi of specimens treated with thiourea (0.03%) were larger and maintained functional activity for a much longer period of time than did those of corresponding controls. Degeneration, although delayed, did occur, but was not as complete as in control specimens.

3. Growth and differentiation of surrounding tissues and organs and lack of blood supply seem to be important in bringing about degenerative changes in thiourea-treated animals.

4. Rapid degeneration of pronephroi in specimens treated with thyroxin (one part in 10 million) during accelerated metamorphosis

indicates the possibility of a direct role of the thyroid in bringing about pronephric degeneration.

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STUDIES ON TUMOR FORMATION IN NICOTIANA HYBRIDS

1. The Classification of the Parents Into Two Etiologically Significant Groups*

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Introduction

Kostoff (1930) reported that certain hybrids among species of the genus *Nicotiana* grew normally until the onset of flowering, at which time they proceeded to form tumors, especially on the inflorescences and toward the base of the stem. A considerable number of further such interspecific tumorous crosses became known through the work of subsequent investigators (Izard, 1952; Kehr, 1951; Kostoff, 1935, 1943; Whitaker, 1934). Kehr and Smith (1954) compiled a list of such hybrids and discussed the various theories proposed to account for the formation of these tumors. Suffice it to say here that the tumors are held to be of genetical origin. They are thus thought to arise in response to stimuli that are part of the organism itself, rather than in response to external stimuli (infective agents) as is the case for the other two well known plant neoplasms (crown gall, Black's wound tumor).

Kehr and Smith (1954) stressed that *Nicotiana langsdorffii, N. glauca, N. rustica* and *N. alata* enter into more tumorous hybrid combinations than other *Nicotiana* species. Accordingly, they considered these species the most effective tumor producers. It must be recognized, however, that the number of tumorous hybrid combinations into which a given species enters (the criterion of the above classification) is a function of two independent sets of parental properties: 1) the range of species with which the *Nicotiana* species can be crossed; 2) the potentiality of the *Nicotiana* species to cause tumor

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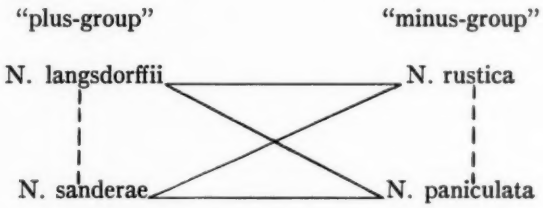
formation upon hybridization with other species. Of these two sets of parental properties only the second is directly related to the process of tumor formation. As regards tumor formation, the classification is therefore of limited value. The question arises whether the parents of tumorous *Nicotiana* hybrids may be classified to provide information that reflects solely their ability or inability to cause tumor formation upon hybridization with other *Nicotiana* species.

The six possible hybrid combinations among the species *Nicotiana langsdorffii, N. rustica, N. sanderae* and *N. paniculata* may be considered in this connection. The pertinent information may be gleaned from the results of Kostoff (1930) who lists four of them among the tumorous combinations. The remaining two crosses, though —*N. paniculata* x *N. rustica* and *N. sanderae* x *N. langsdorffii*—he describes as being nontumorous. This information may be represented by the scheme below which symbolizes tumorous hybrid combinations by solid lines and nontumorous combinations by stippled lines.



It shows that the four parents may be separated into two groups in such a way that hybrids between a species of one group with a species of the other group are tumorous, while hybrids between species that belong to the same group are without tumors. One of these groups contains *N. sanderae* and *N. langsdorffii* (tentatively and arbitrarily called the "plus-group"), while the other group comprises *N. paniculata* and *N. rustica* (tentatively and arbitrarily called the "minus-group").

In view of this, the hypothesis is put forward that other parents of tumorous hybrids may also be classified either as plus-species or as minus-species. Thus a parent would be assigned to the plus-group if its hybrids with the already established members of the minus-group were tumorous while its hybrids with the already established members of the plus-group were without tumors. Conversely, it would be assigned to the minus-group if its hybrids with the already established

members of the plus-group, but not those with members of the minus-group, were tumorous.

Confirmation of this hypothesis would lead to the conclusions that *a*) the critical contribution of minus-parents to tumor production in hybrids differs from that of plus-parents, and *b*) the critical contributions made by parents of the same group are identical. These simple conclusions would be the starting point of any attempt to explain tumor production in the hybrid in terms of parental characteristics whether such attempts be made on a genetical, physiological, or biochemical level.

It is proposed, therefore, to test the validity of the above hypothesis on further hybrid combinations among the *Nicotiana* species that were previously shown to be parents of tumorous hybrids.

Experimental Section

The hypothesis outlined in the Introduction was tested experimentally by producing a considerable number of hybrids among those *Nicotiana* species that were cited by earlier investigators as being a parent of at least one tumorous hybrid. The investigations further involved the species *N. bonariensis* and *N. forgetiana* because they belong to the section Alatae of genus *Nicotiana* which comprises some of the most effective parents of tumorous hybrids. Special stress was laid upon combinations which the hypothesis predicts to be nontumor-forming. Such crosses are of equal importance for an experimental test of the hypothesis. In the literature, pertinent information is available for only a few of them (Kostoff, 1930).

The decision on tumor formation was, where possible, based on 30 plants; the exact number follows the designation of the hybrid in parentheses. Observations ranged from the early seedling stage to five months beyond the onset of flowering. Examination of the roots occurred at three intervals: while the seedlings were transferred from the seedbed to 4-inch pots, from 4-inch pots to 6-inch pots, and again upon termination of the experiment. Failure to achieve a cross is marked by the annotation "unsuccessful".

N. bigelovii x *N. bonariensis*—Excellent germination; however, the seedlings became yellowish soon after germination and died before the primary leaves had formed. No evidence of tumor formation.
N. bigelovii x *N. forgetiana*—Behaved as the aforementioned hybrid.
N. debneyi x *N. bonariensis*—Unsuccessful.
N. debneyi x *N. forgetiana*—Unsuccessful.
N. debneyi x *N. suaveolens* (3)—Nontumorous.

N. debneyi x *N. tabacum*—Excellent germination. However, the seedlings died soon after they had formed the primary leaves and without any evidence of tumor formation.

N. forgetiana x *N. alata* (26)—Nontumorous.

N. langsdorffii x *N. bonariensis* (30)—Nontumorous.

N. langsdorffii x *N. forgetiana* (25)—Nontumorous.

N. longiflora x *N. alata* (30)—Nontumorous.

N. noctiflora x *N. alata* (3)—Nontumorous.

N. paniculata x *N. bigelovii* var. *multivalvis* (30)—Nontumorous.

N. paniculata x *N. bonariensis*—Unsuccessful.

N. plumbaginifolia x *N. bonariensis* (27)—Nontumorous.

N. plumbaginifolia x *N. forgetiana* (23)—Nontumorous.

N. rustica x *N. bigelovii* var. *multivalvis* (30)—Nontumorous.

N. rustica var. *humilis* x *N. bonariensis*—About 40 to 50 per cent of the seedlings began to form tumorous outgrowths a few days after germination, at the time that the primary leaves were formed or soon thereafter (Fig. 2 a-d). The tumors originated in most cases from the axils of the cotyledons and their bulk soon surpassed that of the organized tissue. None of the afflicted seedlings survived. The remaining 50 to 60 per cent of the seedlings lacked any visible symptoms of abnormal growth. Of about 200 such unafflicted seedlings, all but 3 died soon after they had formed the primary leaves. The 3 survivors grew at an extremely slow rate and their leaves were narrow and stringy. Abnormal growths did not appear until about a month after the onset of flowering. They were of the teratoma-type (2-3 per plant) and were found only on the inflorescences.

N. rustica var. *humilis* x *N. forgetiana*—Tumor formation set in at the same early stage of development, and at the same sites, as on the aforementioned hybrid (Fig. 3 a-d). However, all seedlings formed tumors and, probably as a consequence of this, none survived. Instead, the rapid growth of the tumors led to a breakdown of normal development in most seedlings even before they had formed the secondary leaves.

N. rustica var. *humilis* x *N. paniculata* (20)—Nontumorous.

N. rustica x *N. suaveolens*—Unsuccessful.

N. sanderae x *N. alata* (30)—Nontumorous.

N. sanderae x *N. forgetiana* (30)—Nontumorous.

N. sanderae x *N. plumbaginifolia* (12)—Nontumorous.

N. suaveolens x *N. bigelovii* var. *multivalvis* (30)—Nontumorous.

N. suaveolens x *N. bonariensis* (3)—Abnormal growths arose around the junctures of root and shoot on each of the 3 seedlings when they had reached a total length of about 3 cms. Upon reaching a diameter of about 1½ cms. (see Fig. 1) they began to dry out, became brownish and shrivelled, finally falling off the plant. New outgrowths arose at the same site. These died also, and the cycle was repeated several times as the hybrids grew to maturity. Abnormal growths did not arise at other sites except on the inflorescences about a month after the onset of flowering. Whereas the abnormal growths around the basis of the stem lacked any external sign of organization, those on the inflorescences were without exception of the teratoma type.

N. suaveolens x *N. forgetiana*—Unsuccessful.

N. suaveolens x *N. glauca* (30)—Nontumorous.

N. suaveolens x *N. longiflora* (30)—All plants of this hybrid were of normal appearance until about 4 months after the onset of flowering. Abnormal growths of the teratoma type (1 to 3 per plant) then began to arise on the inflorescences of 14 plants (see Fig. 4), while the remaining individuals lacked any sign of tumor formation even 4 months thereafter.

N. suaveolens x *N. plumbaginifolia* (30)—Nontumorous.

N. tabacum x *N. bigelovii* var. *multivalvis* (30)—Nontumorous.

N. tabacum x *N. bonariensis*—Unsuccessful.

N. tabacum x *N. forgetiana*—Unsuccessful.

N. tabacum x *N. plumbaginifolia* (3)—Nontumorous.

FIGURE 1
Tumor formed on the hybrid *Nicotiana suaveolens* x *N. bonariensis*.

FIGURE 2 a-d
Tumor formation on seedlings of the hybrid *Nicotiana rustica* var. *humilis* x *N. bonariensis*.

FIGURE 3 a-d
Tumor formation on seedlings of the hybrid *Nicotiana rustica* var. *humilis* x *N. forgetiana*.

These results, together with those obtained by earlier investigators, are summarized in Tables 1 and 2 (tumorous and nontumorous crosses, respectively). Figure 5 is a schematic representation of both the tumorous and the nontumorous hybrid combination (see legend).

TABLE 1
Tumorous Hybrids[1]

Female parent	Male parent	Authority
N. bigelovii[2]	*N. glauca*	Hitier & Izard, 1951
N. bigelovii	*N. sanderae*	Kostoff, 1935
N. bigelovii var. *quadrivalvis*	*N. sanderae*	Whitaker, 1930
N. debneyi[2]	*N. plumbaginifolia*	Kehr, 1950
N. glauca	*N. langsdorffii*	Kostoff, 1930
N. glauca	*N. longiflora*	Kostoff, 1943
N. glauca	*N. plumbaginifolia*	Kostoff, 1943
N. glauca[2]	*N. sanderae*	Brieger & Forster, 1942
N. glutinosa[3]	*N. glauca*	Hitier & Izard, 1951
N. miersii	*N. langsdorffii*	Kostoff, 1943
N. langsdorffii[4]	*N. glauca*	Kostoff, 1943
N. paniculata	*N. langsdorffii*	Kostoff, 1930
N. paniculata	*N. sanderae*	Kostoff, 1930
N. plumbaginifolia[4]	*N. glauca*	Kehr, 1950
N. rustica	*N. alata*	Kostoff, 1930
N. rustica var. *humilis*	*N. bonariensis*	author's result
N. rustica var. *humilis*	*N. forgetiana*	author's result
N. rustica	*N. langsdorffii*	Kostoff, 1930
N. rustica	*N. noctiflora*	Kostoff, 1935
N. rustica	*N. sanderae*	Kostoff, 1930
N. rustica var. *humilis*	*N. tabacum* var. *sanguinea*	Kostoff, 1930
N. suaveolens	*N. bonariensis*	author's result
N. suaveolens	*N. langsdorffii*	Kostoff, 1943
N. suaveolens[2]	*N. longiflora*	author's result
N. suaveolens	*N. sanderae*	Kehr, 1950
N. tabacum var. *sanguinea*	*N. alata*	Kostoff, 1930
N. tabacum var. *wiganoides*	*N. sanderae*	Kostoff, 1930

[1] Tumorous hybrids combining the genomes of more than two parents (see Kehr and Smith, 1954) are not listed because it cannot be readily decided how many of the participating genomes are necessary for tumor production.
[2] Tumors appear in only part of the hybrid population.
[3] Not entered into Figure 5 because it is only *N. glutinosa*—hybrid for which information regarding tumor formation is available.
[4] Reciprocal combinations of hybrids listed elsewhere in the Table.

The parents are spaced along the vertical line at arbitrarily equal distances; their positions along the line represent, very tentatively, their degrees of plus-ness or minus-ness, respectively.

It was stressed earlier that a majority of the tumorous hybrid combinations delay the formation of tumors until after they have reached the flowering stage. Accordingly, crosses which fail to survive beyond the seedling stage must remain unclassified if they fail to form tumors

TABLE 2
Nontumorous Hybrid Combinations (Among Parents of Tumorous Hybrids)

Female parent	Male parent	Authority
N. alata	*N. forgetiana*	author's result
N. debneyi	*N. suaveolens*	author's result
N. glauca[1]	*N. sanderae*	Kostoff, 1930
N. langsdorffii	*N. bonariensis*	author's result
N. langsdorffii	*N. forgetiana*	author's result
N. longiflora	*N. alata*	author's result
N. noctiflora[2]	*N. alata*	author's result
N. paniculata	*N. bigelovii* var. *multivalvis*	author's result
N. paniculata	*N. glauca*	Kostoff 1930
N. plumbaginifolia	*N. bonariensis*	author's result
N. plumbaginifolia	*N. forgetiana*	author's result
N. plumbaginifolia	*N. langsdorffii*	author's result
N. rustica	*N. bigelovii* var. *multivalvis*	author's result
N. rustica	*N. paniculata*	Kostoff, 1930
N. rustica var. *humilis*	*N. paniculata*	author's result
N. sanderae	*N. alata*	author's result
N. sanderae	*N. forgetiana*	author's result
N. sanderae	*N. langsdorffii*	Kostoff, 1930
N. sanderae	*N. plumbaginifolia*	author's result
N. suaveolens	*N. bigelovii* var. *multivalvis*	author's result
N. suaveolens	*N. glauca*	author's result
N. suaveolens	*N. plumbaginifolia*	author's result
N. tabacum	*N. bigelovii* var. *multivalvis*	author's result
N. tabacum	*N. glauca*	Kostoff, 1930
N. tabacum	*N. paniculata*	Kostoff, 1930
N. tabacum[2]	*N. plumbaginifolia*	author's result

[1] This hybrid was later reported to be tumorous by Brieger and Forster (1942).
[2] Results not entirely certain because not enough hybrid plants could be obtained.

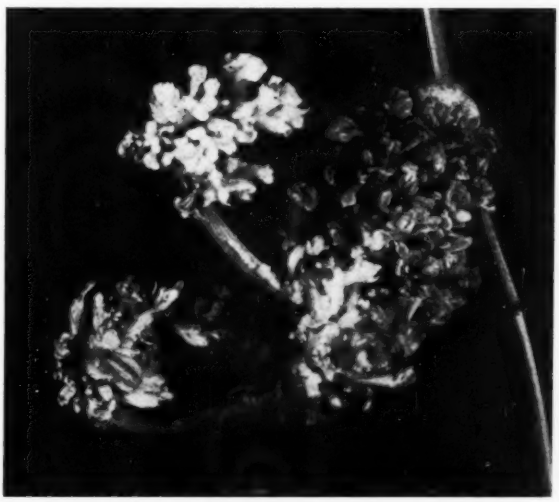
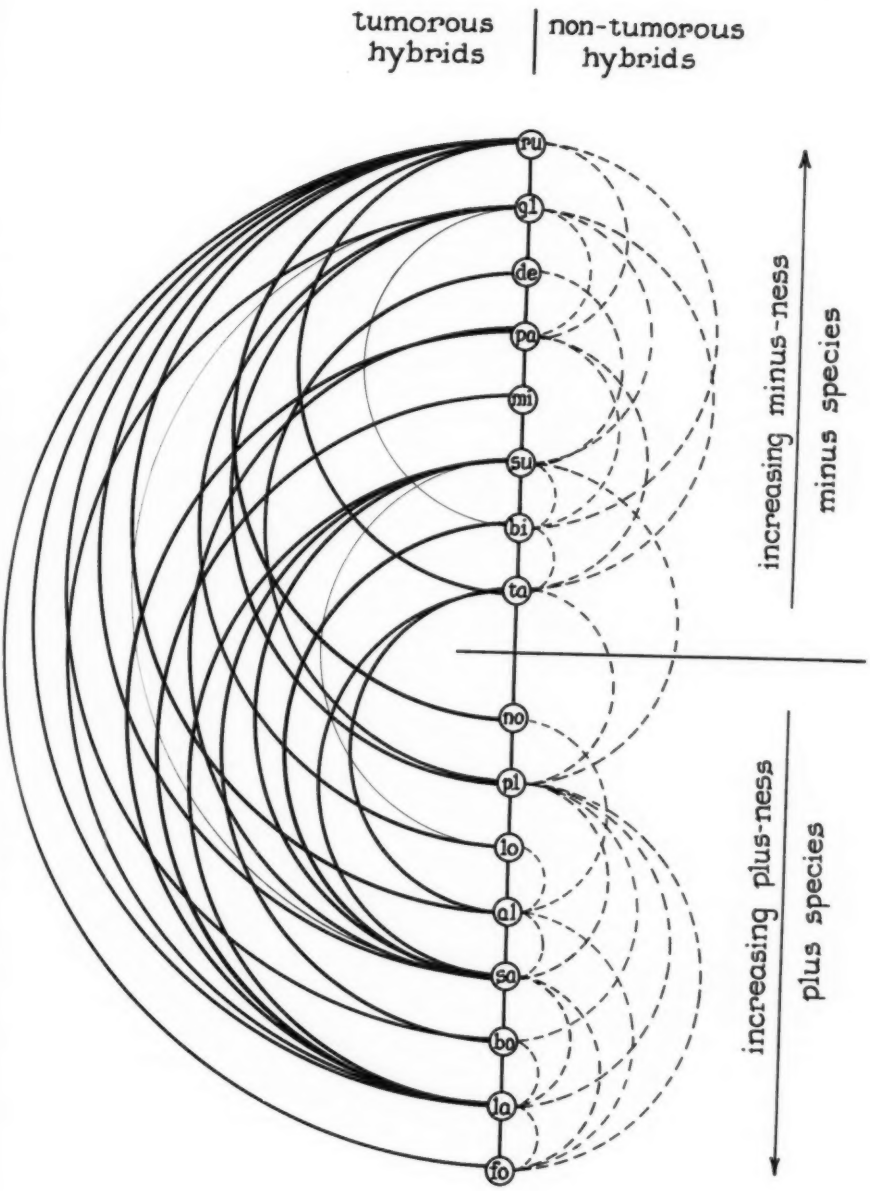


FIGURE 4
Teratoma on the hybrid *Nicotiana suaveolens* x *N. longiflora*.

FIGURE 5

ru	*N. rustica*	no	*N. noctiflora*
gl	*N. glauca*	pl	*N. plumbaginifolia*
de	*N. debneyi*	lo	*N. longiflora*
pa	*N. paniculata*	al	*N. alata*
mi	*N. miersii*	sa	*N. sanderae*
su	*N. suaveolens*	bo	*N. bonariensis*
bi	*N. bigelovii*	la	*N. langsdorffii*
ta	*N. tabacum*	fo	*N. forgetiana*

——— tumorous hybrids (all individuals of the hybrid population).
——— tumorous hybrids (not all individuals of the hybrid population).
– – – – – nontumorous hybrids.


tumorous hybrids
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increasing plus-ness
plus species

during their brief span of life (*N. bigelovii* x *N. bonariensis, N. bigelovii* x *N. forgetiana, N. debneyi* x *N. tabacum*). They are, therefore, not listed either in the tables or in Figure 5.

An evaluation of the results in terms of the above hypothesis is deferred to the Discussion.

Discussion

The hybrids reported in the Experimental Section involve a total of 15 species, all but 2 of which (*Nicotiana bonariensis* and *N. forgetiana*) have been previously reported as parents of at least one tumorous hybrid. Information on tumor formation is now available for 49 crosses (24 by the author, 25 by other investigators), *i.e.,* about 30 per cent of the theoretically possible 136 combinations among the 16 species that were reported to be parents of tumorous *Nicotiana* hybrids. Whereas some further crosses may be obtained, a majority of the remaining ones are not feasible. Since the choice of the crosses was without any known bias, the available information should provide a fair test of the postulate that the parents of tumorous hybrids may be separated into plus- and minus-species by means of the criteria, and with the implications, set forth in the Introduction.

Application of these criteria leads to a plus-group containing *Nicotiana forgetiana, N. langsdorffii, N. bonariensis, N. sanderae, N. alata, N. longiflora, N. plumbaginifolia,* and *N. noctiflora.* The minus-group, in turn, is found to comprise *N. rustica, N. glauca, N. debneyi, N. paniculata, N. miersii, N. suaveolens, N. bigelovii,* and *N. tabacum.*

The results, together with those of earlier investigators, are summarized by Figure 5 which shows that 45 out of the 49 hybrids behave as predicted, *i.e.,* they are tumorous only if they are hybrids between a plus-species and a minus-species. Of the 4 exceptions, *N. rustica* var. *humilis* x *N. tabacum* var. *sanguinea* and *N. bigelovii* x *N. glauca* form tumors contrary to the hypothesis because they are crosses between parents that in other hybrid combinations behave as minus-parents. Conversely, *N. suaveolens* x *N. plumbaginifolia* and *N. tabacum* x *N. plumbaginifolia* are without tumors even though they are predicted by the hypothesis to be tumorous.

While there are 4 exceptions, the large degree of compliance nevertheless indicates that the hypothesis has a basis in fact. The exceptions may be rationalized by drawing an analogy with the phenomenon of relative sex (Hartmann, 1956). This phenomenon denotes the existence of gradations of "mating strength" among the gametes of

either sex. In consequence of this (or more directly in consequence of the physiological mechanism that determines sex), a gamete that behaves female in most cases may act male instead if its female tendency is weak and if brought in contact with a gamete the female tendency of which is strong. Such "exceptions" to mating do not deny the existence of the two sexes and the necessity of the critical differences between them for the production of a zygote. Similarly, the 4 exceptions to tumor formation in *Nicotiana* hybrids need not invalidate the proposed separation of the parents into plus- and minus-species, or deny the necessity of the critical differences between the parents of a tumorous hybrid for tumor production.

Figure 5 shows irregularities of still a different kind. Thus, the hybrid *Nicotiana suaveolens* x *N. sanderae* forms tumors only on about one out of two individuals of the hybrid population. It must, for this reason, be considered less tumorous than the hybrids *N. tabacum* x *N. sanderae* and *N. bigelovii* x *N. sanderae* (tumors on all individuals and on all parts of the plant) even though the reverse is expected. The same type of irregularity is encountered with the weakly tumorous hybrid *N. bigelovii* x *N. glauca*[1] on one hand and the non-tumorous hybrids *N. rustica* x *N. bigelovii* and *N. tabacum* x *N. glauca* on the other hand (see Figure 5). The following considerations are of pertinence in this connection: The expression of the genes directly concerned with tumor formation in the hybrid is likely to be a function of modifier genes. Accordingly, the expression of the tumorous potentiality, *e.g.* of *N. sanderae*, must be considered variable depending on the *Nicotiana* species with which it is crossed. Whereas such considerations may account for the mentioned irregularities, future results may nevertheless lead to a modification of the proposed scheme.

A discussion of the genetical relationships among the parents of tumorous hybrids is of interest. The plus-group contains the seven species making up the section Alatae (*N. alata, N. langsdorffii, N. sanderae, N. forgetiana, N. bonariensis, N. plumbaginifolia,* and *N. longiflora*). In addition it appears to include *N. noctiflora* of the section Noctiflorae which is more closely allied to the Alatae than to any of the other numerous sections of the genus *Nicotiana*. Thus

[1] The author could not find any tumors on this hybrid even though the roots, where Izard (1952) observed the tumors, were examined at four different stages of development in a total of 75 plants.

this group behaves as a unit not only regarding tumor formation in hybrids but also on the basis of genetical relationships.

The minus-group does not contain representatives of the two sections found in the plus-group. Instead it comprises (sections of the genus *Nicotiana* in brackets): *N. glauca, N. paniculata* [Paniculatae], *N. rustica* [Rusticae], *N. tabacum* [Genuinae], *N. bigelovii, N. miersii* [Acuminatae], *N. suaveolens, N. debneyi* [Suaveolentes]. It thus contains species of five different sections most of which are but distantly related to each other and to the sections making up the plus-group. Thus, unlike the plus-group, the minus-group is heterogeneous on the basis of genetical relationships.

This analysis recalls the statements by Whitaker (1934) that tumor formation occurs mainly between 9 chromosome-species of the genus *Nicotiana* and certain other *Nicotiana* species. It anticipates, to some extent, the separation of the parents into two groups. Figure 5, though, shows that the group containing the 9-chromosome species (*N. forgetiana, N. langsdorffii, N. bonariensis, N. alata, N. sanderae*) comprises in addition a 12-chromosome species (*N. noctiflora*) and two 10-chromosome species (*N. plumbaginifolia* and *N. longiflora*).

The fact that the parents of tumorous hybrids may be separated into two groups led to the conclusion that tumor formation in the hybrid is a function of two factors or factor complexes, one being dependent on the genome of minus-species, the other on the genome of plus-species. Progress toward defining these parental contributions on a genetical level was made by Kostoff (1935) and especially by Kehr and Smith (1954). While primarily genic in nature, the critical contributions of the parents to tumor production in the hybrid should be reflected also in parental metabolism. Their identification on a physiological level will make it possible to explain tumor production in the hybrid in terms of parental metabolism.

Attainment of such a goal is hampered by the lack of definitive information concerning the basic difference between normal plant tissues and their neoplastic derivatives. Nevertheless, the ability of crown-gall tissue (Braun and White, 1943; White and Braun, 1942) and of virus-tumor tissue (Black, 1947) to grow in the absence of added auxin must be considered a distinctive characteristic of these neoplasms. Auxin-independence is displayed also by the excised stem tissues of the tumorous hybrids *N. langsdorffii* x *N. glauca* (White, 1939), *N. langsdorffii* x *N. suaveolens,* and *N. bonariensis* x *N. suaveolens,* while the stem tissues of the investigated parents require auxin and in

some cases additional factors (author's results). Thus it appears likely that the acquisition of auxin-independence is important for the transition to neoplastic growth. Indeed the possibility has been increasingly considered that auxin-independence and independence with regard to other growth factors (see Braun, 1957a, 1957b) are the basis of neoplastic growth in plants. If this hypothesis be accepted, then at least one of the critical contributions of the parents must be thought of as leading to the establishment of auxin-independence in the tumorous tissues of the hybrid. Investigations based on this hypothesis, which may be implemented in various ways, are now in progress.

Summary

The hypothesis is proposed that the species involved in the production of tumorous interspecific *Nicotiana* hybrids can be separated into two groups, one called arbitrarily the plus-group, the other the minus-group. Hybrids between a plus-species and a minus-species would be tumorous, while hybrids between plus-species only and hybrids between minus-species only would be without tumors.

Twenty-four hybrids were obtained to test the validity of this hypothesis. Adding the results of earlier investigators, information on tumor formation is now available for 49 hybrids. Of these 49 hybrids, 45 behave as predicted by the hypothesis. The four exceptions notwithstanding, it is argued that the hypothesis has a basis in fact. It was concluded that the critical contribution of the plus-parent to tumor production in the hybrid differs from the critical contribution of the minus-parent. This conclusion is considered the basis of any attempt to explain tumor production in the hybrid in terms of parental characteristics whether such attempts be made on a physiological, biochemical, or genetical level. The possible nature of these different critical contributions is discussed.

Based on the above hypothesis, four new tumorous hybrids were produced: *N. rustica* x *N. forgetiana*, *N. rustica* x *N. bonariensis*, *N. suaveolens* x *N. bonariensis*, and *N. suaveolens* x *N. longiflora*. Tumor formation in the seedlings of *N. rustica* x *N. forgetiana* and of *N. rustica* x *N. bonariensis* sets in very early, at the time that the primary leaves are formed or shortly thereafter. They died as a result of tumor formation. These two tumorous hybrids thus differ from those described by earlier investigators which proceed to form tumors at a much later developmental stage, mostly well after the onset of flowering.

Acknowledgments

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THE ACHROMATIC SPINDLE AND TELOPHASE NUCLEI AS INDICATORS OF PROTOPLASMIC POISONING

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SOME years ago we studied the effects of the organic mercurial phenyl mercuric hydroxide (PMOH) on mitosis in Mus corneas (7) and in Allium root tips. The cornea is a very handy tissue for the experimental cytologist, for the component cells possess great similarity and it "is perhaps one of the simplest organized structures in the mammalian body" (4). Recent studies on this tissue have been reviewed by Friedenwald (4), and Buschke, Friedenwald and Fleischman (2) have published some photomicrographs. They found that the normal period for mitosis was an hour, and that when one eye was treated with colchicine, there was some effect on the other cornea. In our experiments the right eye of a mouse was irrigated every hour for six hours with an aqueous solution of the mercurial in a concentration of one part in 100,000 of distilled water to one in 3,000 ($34 - 1120 \times 10^{-6}$ M). The left cornea acted as a control and sometimes showed a slight arresting effect on mitosis.

An hour after the last treatment the animals were killed by etherization and the eyes irrigated with Bouin's fixative. The corneas were excised, fixed in Bouin's, stained with Hematoxylin, and mounted whole. Counts were made of the stages of mitosis and the effect of the lowest concentration of PMOH was found to be an arrest in telophase (Exp. 2.0%, Control 41.0%) and a marked reduction in anaphase figures (7). After PMOH 1 : 25,000 there was an increase in prophases (Exp. 28.3%, Controls 14.8%) and in metaphases (Exp. 21.8%, Controls 12.7%). With higher concentrations there was a gradual destruction of the achromatic spindle in some dividing cells;

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there were fewer anaphases and telophases than in the control and arrest in metaphase (Metaphase proportions after 1: 10,000 to 1: 3,000; Exp. 94.6-66.5%; Controls 32.7-45.3%). The rest of the treated dividing cells were about equally distributed among prophase, anaphase, and telophase. In all treated corneas the mitotic index was equal to, or higher than, that of the control. One of us has reported the gradual destruction of the achromatic spindle by high dilutions of PMOH and of basic phenyl mercuric nitrate (PMN) in the onion root tip meristem (8, 9, 10, 11). Dividing cells were arrested in metaphase as with colchicine, a condition therefore called c-mitosis. The concentration of the poison that penetrates the protoplasts is conjectural and was estimated at about one-tenth of the strength used in an hour's exposure in the root tip cells (8, 9).

Because of the renewed interest in the mechanisms of mitosis (12, 17) our preparations were examined again for early symptoms of protoplasmic poisoning in telophase. Some time ago Ehrenberg (3) found that the first step in c-mitosis in willow root tips was an increase in the curvature of the spindle fibers, which resulted in a shorter distance between the poles and slowed down the movement of the chromosomes at anaphase.

Telophase in Mouse Cornea after PMOH

Measurements were made by one of us (AAK) in cornea telophase nuclei of:

(a) Distance between daughter nuclei, or inner caliper.
(b) Distance between outer surface of daughter nuclei, or outer caliper.

The former indicated the distance the arms of the sister chromatids separated from each other in ana-telophase, the latter the length of the achromatic spindle from pole to pole. Cells were measured in which the daughter nuclei had formed membranes and cleavage was usually beginning; the first ten telophase nuclei found with a horizontal spindle plane only were chosen. Both dimensions were reduced more than 10% in the treated mouse cornea cells, except after 1: 50,000 and 1: 25,000. However the latter cells were slowed down in prophase, metaphase and in anaphase, some blocked c-metaphases were present and some of the telophases observed may have been formed before much poison penetrated. The average from ten nuclei of each dimension of treated and control corneas is given in Table I.

Two of the controls were poorly stained and could not be measured accurately.

TABLE I

Mouse Corneas after PMOH

Dimensions in early telophase nuclei in micra.

Measurements made with ocular micrometer under oil immersion 97X; oc. 10x.
Mean of ten nuclei given. Fixed one hr. post. 5 hourly irrigations.
A = distance between daughter nuclei, inner caliper.
B = outer caliper between daughter nuclei, or distance between poles. length of achromatic spindle.

Concentration PMOH	Control		Experimental		% reduced in Exp.	
	A	B	A	B	A	B
1: 100,000 — 3.4 × 10^{-5}M	7.1	12.5	6.3	10.9	11.2	12.8
1: 50,000 — 6.8 × 10^{-5}M	—	——	6.8	12.9	——	——
1: 25,000 — 1.4 × 10^{-4}M	5.9	11.5	5.7	11.7	3.4	0
1: 10,000 — 3.4 × 10^{-4}M	6.6	12.1	3.9	9.8	40.9	19.0
1: 5,000 — 6.8 × 10^{-4}M	—	——	2.9	8.9	——	——
1: 3,000 — 11.2 × 10^{-4}M	5.8	10.7	1.9	7.1	67.2	30.3
Average	6.4	11.7	4.6	10.2	25.0	12.8

A definite retarding effect of the mercurial on the separation of the daughter nuclei and a diminished spindle length is shown (Table I). The shape of the spindle fibers was not observable in the cornea nor in the root tip preparations.

Telophase in Root Cells after Respiratory Poisons

These investigations were originally made when our colleagues were testing the effects of organic mercurials on some of the enzymes of the respiratory chain (5). They were thought to be somewhat specific for the —SH dependent enzymes, but have since been found to be nonspecific inhibitors on enzymes and to cause denaturation of protoplasmic proteins (16). Some other enzymic poisons were also tested on the growing root tips of *Allium Cepa* L., potassium cyanide (which inhibits several iron dependent oxidases and has a profound effect on many biological systems) and sodium fluoride (which suppresses aerobic glycolysis). Two narcotics were also used: phenyl urethane (ethyl carbanilate) and magnesium sulphate. Treatments were given in tap water for short periods of time and were below the threshold for nuclear destruction, although some (Table II) were lethal for the more sensitive region of cell elongation (8, 9). The root tips were fixed in acetic-alcohol and stained in squash preparations. Recently the slides were reexamined for effects in telophase and a distinct tendency was evident for a shortening of the spindle

and a decrease in the distance between the daughter nuclei, see Table II.

TABLE II

Onion Root Meristems after short exposure to poisons in Tap Water.
Dimensions in Telophase nuclei in micra. Obj. 40x; Oc. 10x.
Range in ten nuclei, A—between daughter nuclei; B—length of spindle.

Exp.	Treatment & Time in min.	A	B	Gross effect on zone of cell elongation
1-7	*Tap Water—Controls*	7.4-12.9 5.8-17.1	19.4-30.7 22.6-33.3	————
1.	KCN 36 × 10^{-5}M — 60′	6.5- 9.6	17.8-27.5	Slight distortion
2.	KCN 0.2% (3.07 × 10^{-2}M) 120′	3.2-11.3	11.3-19.6	lethal (MLD 1 hr.)
3.	KCN 0.4% (6.14 × 10^{-2}M) 120′	3.9- 9.7	14.5-20.7	lethal (MLD 1 hr.)
4.	NaF 0.5M — 90′	1.6- 7.4	12.9-25.8	lethal (MLD 1 hr.)
5.	$MgSO_4$ 0.5M — 120′	3.2- 9.7	12.3-22.6	none
6.	$C_6H_5NHCOOC_2H_5$ 2.0% (12 × 10^{-2}M) 240′	0 - 9.7	16.8-26.0	Slight distortion (MLD 4 hrs.)
7.	$C_6H_5NHCOOC_2H_5$ 3.0% (18 × 10^{-2}M) 40′	2.6-13.9	19.4-22.6	none
8-10	*Tap Water Control*	6.5-10.7	18.1-24.2	————
8.	PMN 1: 25,000 (6.8 × 10^{-5}M) 100′	4.8- 8.1	19.4-24.2‡	lethal*
9.	PMN 1: 50,000 (3.4 × 10^{-5}M) 60′	2.6- 9.0	16.8-22.6‡	lethal*
10.	PMOH 1: 100,000 (3.4 × 10^{-5}M) 69′	1.6-13.9	12.9-23.2‡[v]	lethal*

* MLD for 60′ for elongating cells in mercurials: 7-8 × 10^{-6}M.
‡ Some arrest in metaphase at end of treatment. Telophases may represent cells caught in mitosis at beginning.
[v] Full c-mitosis (spindles destroyed) 5 hrs. after return to tap water.

Because the root-tip cells vary in the different zones of the meristem (3), the range of the two dimensions in ten telophase nuclei from the central region is given, instead of the average values. The relatively large size of the plant cell is remarkable. All bulbs used were of the same variety (8) and dimensions in telophase in three normal roots from bulbs grown in tap water at the same time are given.

These observations are of limited scope but are presented as a suggestion for symptoms of early protoplasmic poisoning, also as a method for investigating the mechanisms of mitosis. A specific enzymic poison is not yet known (17), nor agents that affect only one part of the cell or of mitosis.

Some special effects on the telophase, as well as three on the resting nuclei, produced by the individual treatments, are shown in Table III. When two or more treatments with an agent were given, if the special effect was noted only after one, the effective concentration is noted. A tilted, diagonal spindle was often observed after PMOH and PMN in high dilutions (9, Fig. 2a) and could be a result of re-

duced cytoplasmic viscosity. Diagonal cell plates (new wall between daughter nuclei) were observed after phenyl urethane and sodium fluoride. The spindle fibers may be deformed, or twisted, so that one or both groups of chromosomes at early telophase lie in a plane at right angles to the equator. This gives a "star" effect similar to that figured by Östergren (14) in onion root after aminopyrin and was seen after the weaker phenyl urethane and PMOH (6.8×10^{-5} M/1hr.); no amphiasters could be detected.

TABLE III
Onion Root Tip Meristems

Specific effects of poisons in telophase and interphase after treatments listed in Table II.

±1 or 2 seen + below 50% ++ about 50% +++ complete.

Phenomenon	KCN	NaFl	Mg_2SO_4	Phenyl Carbomate	PMN & PMOH
1. Spindle abnormalities					
(a) Tilted or diagonal	+	+		+	+
(b) Diagonal cell plate & wall		±		±	
(c) Bent fibers; daughter nuclei perpendicular to equator—"Star" anaphase				±(2%)	±
(d) Lopsided; daughter nuclei not parallel	+	±			+
2. Cell plate formation inhibited	+(0.4%)	+++	++	++	++
3. Daughter nuclei barely separate, may touch or overlap		++		+(3%)	±
4. Delayed rounding of daughter nuclear membranes	++				
5. Daughter nuclei at or through plasma membrane	+(0.4%)			+(3%)	
6. Swollen nucleoli in daughter nuclei & interphase nuclei	++(0.4%)				++ (also CO_2 ++)
7. Chromsome bridges late anaphase	+				+
8. Early pyknosis, interphase	++(0.4%)	++	++	+(3%)	
9. Polyploidy				+	+
10. Increased differentiation in meristem				+	+

The phragmoplast or inner spindle (14, Fig. 1), was present in all experiments (even when cell plate formation and cleavage were inhibited), except after the short non-lethal treatment with phenyl urethane at 3% (18×10^{-2} M) and after two hours in the stronger KCN at 6.14×10^{-2} M, which was lethal for elongating cells. Cleavage inhibition may be due to poisoning of the peripheral cytoplasm (1); it is also blocked by adenine and 2,6-diaminopurine (17)

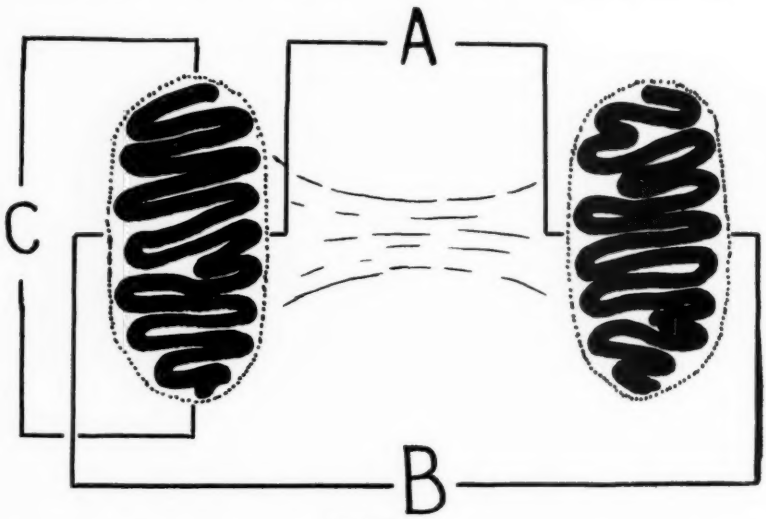

FIGURE 1
Semi-diagrammatic illustration indicating the regions where caliper measurements were made on the dividing cells in early telophase.

Beatty (1) attributed polynuclearity to the fact that the spindle is functioning in inhibited cytoplasm. Close approximation of the daughter nuclei in several treatments gave rise to binucleate cells in the treated root cells. Cell plate formation was suppressed by NaF (Table III) and delayed onset of daughter nuclei organization was characteristic after all the exposures to KCN as with aminopyrin (14). After the strongest KCN treatment the chromosome arms remained visible, although contracted and "vacuolated" and the daughter nuclei remained irregular in outline in late telophase. Delayed onset of daughter nuclei formation with arrest in telophase was

also reported after aminopyrin (14). Hydropic, swollen nucleoli in all organized nuclei, which are prominent after PMOH (9, Fig. 1), were characteristic after the strong KCN poisoning (Table III) and indicate interference with nucleic acid metabolism. Details of these effects and of chromosome despiralization by stronger KCN in onion roots have been given by Levan and Wengenheim (6).

Discussion

The spindle suppression that leads to c-mitosis has been attributed to —SH enzyme inhibition (11, 17). Now that the importance of the glutathione oxidation and reduction cycle in mitosis is better known (12, 17) and disulphide reductase has been found to be essential for division and cell wall formation in yeast (13), this may account for another deleterious effect of these organic mercurials. A concentration of mercury of 10^{-5} M will inhibit glutathione reductase by fifty per cent or more (18). PHOH at 5×10^{-6} M with animal tissue *in vitro* for 1-2 hours, gave a fifty per cent depression in the succinoxidase system; at 7.5×10^{-5} M a fifty per cent depression of the succinic dehydrogenase system (5), and was found to ionize to a phenyl mercuric ion (16). PMOH and also PMN are radiomimetic and mutagenic for plants (9, 10), actions that may be common to several metals that "irreversibly block and alter enzyme systems" (18). Von Rösen also stressed the high toxicity for cell metabolism of complex-forming metals of high atomic number and the importance of determining the intracellular concentrations. He reported radiomimetic effects of both magnesium and sodium in Pisum.

In continuous treatment of onion roots with PMN at 1: 5 million (3.8×10^{-7} M), slow growth continued in clubbed tips with many cells undergoing c-mitosis; however, some functional spindles also were found. After ten days' exposure telophase nuclei showed spindle length (dimension B) from 16.2-25.8 micra, shorter than in a root tip on a control bulb—20 to 35 micra.

A root tip which had been allowed to recover in tap water for a day after five hours exposure to non-lethal urethane (ethyl carbamate $NH_2\ COOC_2H_5$) 0.1% (1.12×10^{-2} M), showed spindle lengths at telophase above normal 26-45 micra. The same range in spindle length was observed in a root tip after a day in a water-saturated atmosphere of CO_2, i.e., extra long spindles in the few telophases remaining.

Summary

After short exposure to phenyl mecuric hydroxide the telophase nuclei in mouse corneas showed a decreased length of achromatic spindle and poor separation of daughter nuclei. The same effects were present in onion root meristems after treatments with two organic mercurials, potassium cyanide, sodium fluoride, magnesium sulphate, and phenyl urethane. It is suggested that these criteria might be useful diagnostic aids for the detection of early protoplasmic poisoning in a variety of embryonic cells.

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INCREASED PARTIAL PRESSURE OF O_2 AND THE ACCELERATION OF DEVELOPMENT IN EARLY CHICK AND FROG EMBRYOS; SIGNIFICANCE OF EARLY PRIMITIVE STREAK ACTIVITIES

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INTRODUCTION

OPINION differs concerning the manner of origin and the functional behavior of the primitive streak in the avian embryo. Moreover, there is a difference of opinion relative to the exact point in development where blastulation ends and gastrulation begins. Opinion also differs with respect to the possibility of homologizing the primitive-streak type of gastrulation found in higher chordates with the gastrulative mechanisms present in lower chordate classes.

This paper presents a comparative study of the effects of increased O_2-pressure on chick and frog embryos. Its purpose in so doing is to determine common reactions to the increased pressure in both embryos. Such reactions would suggest the presence of similar, underlying biochemical systems and, hence, would denote similar stages of development. In addition, observations upon the genesis of the early stages of the primitive streak are described, and a comparison is made of pregastrulative movements in chick and lower chordate embryos.

MATERIALS AND METHODS: THE CHICK EMBRYO

Eggs of single-comb white Leghorn hens within the first week after laying were used. They were kept during the interim between laying and incubation at a temperature of 16° to 18°C. In view of the fact that a steel pressure chamber was employed in these experiments a 4-hour warm-up period was allowed during which the control and experimental eggs were kept in a forced-draft incubator previous to the actual placing of the experimental eggs in the pressure-chamber.

The pressure-chamber used was of 2,700 cm_3 capacity. The degree of pressure was determined by a pressure gauge (Lonergan) permanently installed in the chamber's lid. The pressure gauge reading was standardized by comparisons with a Lonergan inspector's test gauge. The chamber was kept in the forced-draft incubator at all times excepting the short intervals when the chamber was removed to set up the experiment or remove eggs. In consequence, the experimental eggs always were placed in a chamber which was thoroughly warmed to a temperature approximating 38°C. The reason for the warm-up period for the eggs mentioned above is obvious. If cool experimental eggs were placed in such a container while the control eggs were placed in the forced-draft incubator, the experimental eggs would be subjected to the still air within the chamber which was cooled by their own presence. Accordingly, it would take a longer period of time for the experimental eggs to warm up to the incubation level than the control eggs exposed to the warm, moving air of the incubator.

The compressed air and nitrogen employed in these experiments were obtained from the Ohio Chemical and Surgical Equipment Co., a division of Air Reduction Co., Inc. The pumps utilized for compression are water-lubricated, and the air is characterized as 100% pure, compressed in cylinders at Hoboken, N. J. Each cylinder of compressed air or nitrogen was warmed in the forced-draft incubator for several days before use. The nitrogen used was of the prepurified variety.

The terminology relative to pressure employs the word "atmosphere." One atmosphere of pressure is regarded as a sea-level atmosphere of pressure, and hence it approximates 1,033.6 gm. per square cm. This pressure is close to 15 lbs. per square inch. One atmosphere of added pressure signifies one atmosphere added to the normal, single atmosphere present in the chamber immediately after the eggs were inserted. Hence, "one added atmosphere" means that the eggs within the chamber were subjected to two atmospheres. The process of decompression or release of pressure within the chamber was gradually accomplished over a period of ½ hour.

The term "hours of incubation" refers to the total hours of incubation including the 4-hour warm-up period. Six experimental eggs were placed in the chamber and six control eggs were placed in the forced-draft incubator for each individual experiment.

INCREASED AIR-PRESSURE AND THE DEVELOPMENT OF THE EARLY CHICK EMBRYO

Experimental series 1: One added atmosphere for the first 28 hrs.of incubation

Experimental eggs—Total 18

No. of embryos	Stage of development
1	Definitive primitive streak
2	Head process
1	Head fold
2	Two somites
2	Three somites
4	Four somites
4	Seven somites
1	Eight somites
1	Infertile
2	Blastoderm, no streak

Control eggs—Total 18

No. of embryos	Stage of development
1	Broad, fuzzy streak
1	Definitive primitive streak
5	Head process
7	Head fold
1	Two somites
1	Four somites
2	Infertile

These results indicate that there is a definite accelerative effect produced by the one added atmosphere: the youngest in the experimental group is in the definitive, or long, primitive-streak stage, the oldest is in the 8 somite condition and twelve of them are in 2-7 somite stages. In the control group, the youngest is in the broad-streak stage and the oldest is in the 4 somite stage with the greater majority in the head-process and head-fold stages.

Experimental series 2: One added atmosphere for the first 20 hrs. of incubation

Experimental eggs—Total 48

No. of embryos	Stage of development
4	Broad, fuzzy streak
9	Early definitive streak, with groove faint or absent
19	Definitive primitive streak
5	Head process
7	Early head fold
2	Infertile
2	Blastoderm, no streak

Control eggs—Total 48

No. of embryos	Stage of development
6	Broad, fuzzy streak
21	Early definitive streak, groove faint or absent
15	Definitive primitive streak
4	Head process
2	Infertile

The results from this set of experiments show 31 blastoderms of 48 eggs in the definitive primitive streak, head process, and early head fold stages, whereas in the control group 19 blastoderms are in the definitive streak and head process stages. These and the results of Experimental Series 1 indicate that the accelerative effect of the increased atmospheric pressure is upon the definitive streak and later stages and not so much upon the early streak stages. With this evidence in mind a series of experiments were run during the first 14 hrs. of incubation. The results are shown in series 3.

Experimental series 3: One added atmosphere for the first 14 hrs. of incubation

Experimental eggs—Total 48

No. of embryos	Stage of development
3	Pre-streak blastoderms
27	Various stages of early streak
12	Broad, fuzzy streak
4	Broad to definitive streak
2	Infertile

Control eggs—Total 48

No. of embryos	Stage of development
2	Pre-streak blastoderms
30	Various stages of early streak
9	Broad, fuzzy streak
4	Broad, fuzzy to definitive streak
1	Definitive streak
1	Degenerating blastoderm
1	Infertile

The results of this set of experiments upon the earlier phases of primitive streak development shows little effect of the increased atmospheric pressure during this period. In fact, there appears to be a small retarding effect in the experimental eggs. This may be due to the slight lowering of temperature in the experimental group which occurs as the pressure-chamber and eggs are removed from the incubator at the end of the warm-up period when the eggs are placed in the chamber and the increased air pressure is added.

A further inquiry was made concerning the effect of the increased atmospheric pressure upon the later phases of primitive streak development and early body formation. In this series of experiments the eggs were given 34 hours of incubation in addition to the 4-hour warm-up period. The results are given in series 4, below.

Experimental series 4: One added atmosphere for the hours 14-38

Experimental eggs—Total 48

No. of embryos	Stage of development
11	8 somites
17	9-10 somites
12	12-13 somites
5	15-16 somites
3	Infertile

Control eggs—Total 48

No. of embryos	Stage of development
2	3 somites
14	6-7 somites
13	9 somites
12	10-11 somites
3	11-12 somites
4	Infertile

The last two sets of experiments (i.e., experimental sets 3 and 4), suggest strongly that the effect resulting in accelerative development occurs after the broad streak stage, and more probably as the definitive streak emerges and begins to function by segregating rudimentary materials into the future body-region of the embryo. The importance of this fact is discussed below.

Although previous work had indicated (Nelsen '55) that nitrogen has little influence upon development other than to maintain the correct pressure, a separate experimental series was run (series 5) in which one added atmosphere of pre-purified N_2 was substituted for the one added atmosphere of air.

Experimental series 5: One added atmosphere of N_2 for the hours 14-38

Experimental eggs—Total 18

No. of embryos	Stage of development
2	Head fold, 1-2 somites
3	4-6 somites
2	8-9 somites
8	9-11 somites
1	Pre-streak
1	Infertile
1	Degenerate blastoderm

Control eggs—Total 18

No. of embryos	Stage of development
4	3-5 somites
5	7-9 somites
6	10-12 somites
2	Infertile
2	Infertile
1	Undetermined

Increased Partial O_2-Pressure and the Development of the Early Frog Embryo

Methods Used in the Study of the Frog Embryo

Eggs of two species of frogs, Rana pipiens and R. sylvatica were used. The eggs of the latter species were naturally deposited in the last half of March. Only those eggs which were in the early cleavage stages when brought into the laboratory were used. In the case of R. pipiens naturally deposited eggs obtained in the early cleavage stages during the first part of April were used in some of the experiments, and pituitary-induced eggs from females injected with macerated anterior-lobe pituitary substance were employed in other experiments. The pituitary induced eggs were obtained during the months of February and March from females brought into the laboratory shortly before they were injected. All of the female frogs were kept in a cold room at a temperature of from 2-8°C. previous to the injections with the pituitary substance. In all cases, the results from pituitary-induced eggs were not considered as valid experimental results unless the control eggs possessed a cleavage incidence of 90% or better.

Two general types of experimental procedures were employed. In one type of experiment small masses of eggs were placed in stender dishes and covered with tap water to a depth of from 1/8 to 3/8 inches above the surface of the eggs. The stender dishes were then placed at the bottom of a steel chamber of 2,700 cm_3 capacity. Oxygen then was added to the sealed chamber by means of a valve in the chamber's lid. The second type of experimental procedure differed slightly from the one just mentioned. In this procedure egg-masses were suspended in a beaker of water by means of a string in such a manner that some of the eggs were situated immediately below the water's surface while other eggs extended downward from the near-surface eggs for a distance of about 6 cm. The beaker was placed in a pressure chamber and the increased O_2-pressure added. Eggs situated near the surface were thus exposed to a higher concentration of O_2 than eggs in the lower water levels. All eggs under these conditions were exposed to the same total pressure, but those nearer to the surface were exposed to a higher partial pressure of O_2.

Two sets of control experiments were run. One set of controls

consisted of small masses of eggs in a glass-covered finger bowl containing water from the same source as that used for the experimental group. These eggs were surrounded by the normal atmospheric conditions within the refrigerator. A second set of controls was composed of egg-masses within stender dishes. The latter was placed within a pressure-chamber. This second set of controls was exposed to an increased total pressure, but the partial pressure of O_2 was not elevated.

In earlier experiments, added atmospheres of air were used in experiments on the frog embryo. As indicated previously (Nelsen '47), O_2 is the main active element in air which accelerates development. Carbon dioxide also is important, but the possible influence involved in these experiments is slight. (Studies on various amounts of CO_2 in relation to certain fixed quantities of O_2 are in progress. They will be reported later.) For the purposes of this general study on the accelerative effect of O_2 on the early development of frog and chick embryos, equivalent amounts of total added pressure were used to standardize the decompression effects, if any, on both sets of embryos. Hence, 1 atmosphere of air was employed in the chick embryo (= 1/5 atmosphere of added O_2) and 1 atmosphere of O_2 was used in the frog experiments. The frog embryo, in its lower-temperature environment, is much less sensitive to O_2 than the chick embryo in its higher-temperature environment. As 5 added atmospheres of air are necessary to elicit approximately the same acceleration in the frog embryo as 1 added atmosphere of O_2 the latter was used to eliminate the tedious decompression process. Decompression in the case of frog, as in the chick experiments, was carried out gradually over a period of ½ hour.

All of the experiments on frog eggs were made at a temperature of 16-18°C. The pressure chambers and water were kept at this temperature in the refrigerator previous to use in the experiments. The durations of exposure employed on the eggs of R. sylvatica were 15 hrs. and 30 hrs., whereas the exposure-periods employed on R. pipiens were 30 hrs. and 50 hrs. due to the comparably slower rate of development in R. pipiens. The O_2 and N_2 used in these experiments were water-pumped and obtained from the source mentioned above.

EXPERIMENTAL SERIES 1. NATURALLY DEPOSITED EGGS OF R. SYLVATICA.

Initial stage of development and number of eggs used	Type of Experiment	Pressure	Duration of Exposure	Developmental stage when removed from exposure chamber
4-16 cells; 50 eggs	Small masses in stender dishes	1 added atms. of O_2	15 hrs.	43 late blastula; 4 very early dorsal lip; 3 abnormal early to late cleavage stages.
4-16 cells; 50 eggs	Small masses in stender dishes	1 added atms. of N_2	15 hrs.	45 late blastula; 5 abnormal cleavages.
4-16 cells; 50 eggs	Eggs in glass covered finger bowl	No pressure added	15 hrs.	46 late blastula; 2 middle cleavages; 1 late cleavage; 1 cytolyzing.

This experimental series suggests that there is a very slight accelerative effect in development produced by the 1 added atmosphere of O_2. However, as there were only 4 very early dorsal lips the accelerative effect presumably was upon the early gastrulative processes and not upon cleavage and blastulation as such.

EXPERIMENTAL SERIES 2. NATURALLY DEPOSITED EGGS OF R. SYLVATICA.

Initial stage of development and number of eggs used	Type of Experiment	Pressure	Duration of Exposure	Developmental stage when removed from exposure chamber
4-16 cells; 50 eggs	Small masses in stender dishes	1 added atms. of O_2	30 hrs.	2 early neural fold; 6 late neural fold; 32 neural tube closed; 2 tail bud evident; 1 tail bud prominent; 4 middle gastrula; 1 early cleavage; 2 cytolyzed.
4-16 cells; 50 eggs	Small masses in stender dishes	1 added atms. of N_2	30 hrs.	3 early gastrula; 17 middle to late gastrula; 13 yolk plug small; 5 neural plate; 4 neural folds beginning; 3 blastula; 2 cleavage stage; 3 development abnormal.
4-16 cells; 50 eggs	Eggs in glass covered finger bowl	No pressure added	30 hrs.	21 middle to late gastrula; 9 yolk plug small; 7 neural plates; 3 early neural folds; 4 blastula; 3 cleavage stages; 3 cytolyzed.

The data obtained in Experimental Series 2 suggest that the increased O_2-pressure has a pronounced influence upon gastrulative and neuralative processes in the embryo of R. sylvatica.

EXPERIMENTAL SERIES 3. NATURALLY DEPOSITED EGGS OF R. PIPIENS.

Initial stage of development and number of eggs used	Type of Experiment	Pressure	Duration of Exposure	Developmental stage when removed from exposure chamber
8-32 cells; 50 eggs	Small masses in stender dishes	1 added atms. O_2	50 hrs.	3 late gastrula; 3 neural plate; 14 neural fold; 17 neural folds closing; 2 neural folds closed; 2 mid gastrula; 3 blastula; 4 irregular development.
8-32 cells; 50 eggs	Small masses in stender dishes	1 added atms. of N_2	50 hrs.	25 small yolk plug and neural plates; 16 early neural folds; 4 abnormal development; 5 cytolyzed.
8-32 cells; 50 eggs	Eggs in glass covered finger bowls	No pressure added	50 hrs.	27 small yolk plug and neural plate; 18 neural folds; 4 blastula; 1 early cleavage.

The results obtained in Experimental Series 3 in general coincide with those obtained for the eggs of R. sylvatica. The increased O_2-pressure has a definite accelerative effect upon a large majority of the eggs.

EXPERIMENTAL SERIES 4. ARTIFICIALLY INDUCED EGGS OF R. PIPIENS.

Initial stage of development and number of eggs used	Type of Experiment	Pressure	Duration of Exposure	Developmental stage when removed from exposure chamber
One half hr. after artificial insemination at room temp. of 22°C; 48 eggs	Small masses in stender dishes	1 added atms. of O_2	25 hrs.	32 late cleavage; 7 mid-cleavage; 2 about 32 cell stage; 7 irregular cleavage, cytolyzed, or infertile.
As above	Small masses in stender dishes	1 added atms. of N_2	25 hrs.	27 late cleavage; 10 mid-cleavage; 3 early cleavage stages; 8 irregular cleavage, cytolyzed, or infertile.
As above	Eggs in glass covered finger bowl	No pressure added	25 hrs.	39 late cleavage; 4 mid-cleavage; 1 early cleavage; 4 irregular cleavage cytolyzed, or infertile.

The information contained in the above results suggest two conclusions, viz.,

a. There is no apparent acceleration produced by the increased O_2-pressure during the cleavage period, and

b. Exposure to increased pressure produces a higher percentage of irregularly cleaving and cytolyzed eggs.

EXPERIMENTAL SERIES 5. ARTIFICIALLY INDUCED EGGS OF R. PIPIENS.

Initial stage of development and number of eggs used	Type of Experiment	Pressure	Duration of Exposure	Developmental stage when removed from exposure chamber
One half hr. after artificial insemination at room temp. of 22°C; 48 eggs	Small masses in stender dishes	1 added atms. of O_2	50 hrs.	2 late gastrula; 5 neural plate; 21 neural depression shallow; 16 neural depression deep & folds converging; 4 development irregular.
As above	Small masses in stender dishes	1 added atms. of N_2	50 hrs.	16 yolk plugs; 11 neural plate; 13 early neural folds; 8 irregular cleavage, cytolyzed, or infertile.
As above	Small masses in stender dishes	No pressure added	50 hrs.	11 small yolk plugs; 14 neural plate; 11 beginning neural folds; 4 neural depression shallow but folds pronounced; 1 early cleavage; 2 late cleavage; 5 development irregular or infertile.
Approximately 100 eggs	Egg string suspended in beaker	1 added atms. of O_2	50 hrs.	Eggs near surface of water had deep neural folds; Eggs near bottom of beaker had yolk plugs; also few beginning neural folds.

Discussion

The data obtained above indicate that the pre-streak and earlier phases of primitive streak development in the chick embryo, and the cleavage-blastulation period in the frog embryo are not stimulated in their development by exposure to the increased O_2-pressure employed. An accelerative development occurs, however, as the primitive streak assumes its definitive form in the chick blastoderm, and when gastrulation begins in the frog embryo. Increased pressure alone as evidenced by N_2 control exposures (and in the frog by the egg-string-suspension experiments) is not a factor in this accelerative phenomenon. The elimination of pressure as a developmental factor other than that of maintaining an optimum atmospheric pressure has been indicated in the chick (Nelsen '55).

These results suggest that both embryos lack the morphogenetic conditions necessary to accelerate development by utilizing increased amounts of O_2 during the earlier phases of their development. It is not until the later period when cellular materials migrate inward to establish the cellular basis for the developing embryo's body that morphogenetic factors are present which utilize and respond to increased amounts of O_2 by accelerative development. (See also, Malamed, '58, p. 245.) The question therefore arises: does the earlier developmental period characterized by indifference to higher levels of O_2, and the later period marked by O_2-responsiveness correspond to identical general periods of development in both chick and frog embryos? If this question is answered in the affirmative we come to the conclusion that gastrulation in the chick embryo, in a strict sense, as far as the dorsal axial system and embryonic body is concerned, begins not in the pre-streak stage, nor in the early streak stages, but rather in an advanced stage of primitive streak development which corresponds roughly to the termination of the period when the broad streak changes into the definitive-streak condition.

This view of close identity and fundamental similarity of the early phases of development in the chick and frog is contradicted by certain statements in the literature. For example, Lutz, '55, p. 73, describes gastrulation in birds as a two-stage process. The first stage effects the segregation of the entoderm, the second the mesoderm. Rudnick, '44, '55, apparently holds this view. Waddington, '56, p. 168, maintains that mesoderm invagination in the chick embryo begins at the ventral lip of the blastopore although he is careful to state

that this mesoderm "does not form part of the mesoderm of the embryonic axis." If this invagination really marks the beginning of gastrulation it represents a complete reversal of the gastrulative procedure in lower chordates where this phenomenon begins at the dorsal blastoporal area. Hence, there is a conviction among some that gastrulation in birds and mammals cannot be compared closely with that in amphibians because of existing differences. As an illustration, Horstadius, '53 (see Witschi, '53, p. 261) states: "I believe that the differences between different classes of vertebrates are so great, e.g., the primitive streak stage in birds and mammals compared to the gastrulation stages in amphibians, that homologization will be very difficult." However, these so-called differences between vertebrate classes may result from an attempt to homologize superficial morphological events while overlooking basic structural procedures and associated dynamic movements. It will prove fruitful to explore the matter of the developing primitive streak in greater detail.

1. *Mesodermal form-building movements.* One of the precursors to the developing primitive streak are the form-building movements of the presumptive chorda-mesoderm associated with the forward moving hypoblast (Pasteels, '37). Malan, '53, suggested that the presumptive chorda-mesoderm area, according to her careful observations after vital-staining, lies in the pellucid epiblast at its posterior margin. As Malan demonstrated (see also, Pasteels, '53) the chorda-mesoderm moves anteriad to establish the median position of the presumptive area in the posterior half of the area pellucida. Waddington, '33, concluded that the recently formed hypoblast (entodermal) layer is associated with the form-building movements of the epiblast tissue "necessary for the formation of the primitive streak." Evidently, the movement forward of the presumptive chorda-mesodermal area, as described by Malan, is conditioned by inductive influences emanating from the hypoblast layer. As a result, following shortly after the segregation of the entoderm, the general zone of chorda-mesoderm is established in the epiblast layer. The future blastopore (primitive streak) develops in the median line within this zone.

2. *The delineation of the blastopore and development of contact between the chorda-mesoderm and the entoderm.* The dynamic movements of the presumptive entoderm and chorda-mesoderm in the forming chick blastoderm mentioned above mimic to an extent the pregas-

trular movements of cytoplasm which occur at fertilization in some lower chordates, e.g., Styela, Amphioxus, etc. However, the above mentioned pregastrular movements in the chick fail to accomplish one feature found in lower chordates, namely, intimate relationship between the presumptive entoderm and presumptive chorda-mesoderm. In consequence, in the chick blastoderm after the entoderm has been segregated and the chorda-mesodermal zone is moved forward into a dorsal position, contiguity between these two presumptive areas is a relationship still to be established.

One of the difficulties in forming a realistic concept of early primitive streak activities arises from the view that the primitive streak is a gastrulative mechanism from the moment of its origin. A second factor contributing to confusion is failure to appreciate extra-embryonic tissue formation as a special embryonic adaptation not concerned with the immediate problem of the formation of the embryo's body. Hence, extra-embryonic tissue segregation is an added problem which the amniote embryo must face and it should not be permitted to distort those activities of the blastopore (primitive streak) which are comparable to similar activities in the blastopores of lower chordates. A third difficulty in evaluating the processes involved in the primitive-streak type of gastrulation is the close relationship existing between pregastrulation and gastrulation movements, a relationship which is not present in the primitive chordates.

a. *When does the primitive streak become a gastrulative mechanism?* The present author has studied sections of twenty-five developing primitive streaks from the early stages of origin to the broad streak condition and he cannot concur with the view that the streak is concerned with the active gastrulative processes during this young period of its formation. A more detailed and graphic presentation of this study will be presented elsewhere. For the purposes of this discussion certain aspects of this study are presented here.

The primitive streak arises as a thickened, posterior plate of cells at the caudal end of the epiblast near or at the posterior margin of the area pellucida. Its caudal edge may infringe upon the area opaca. The extent of this infringement varies. The posterior plate is a broadened area and in microscopic sections cells can be seen detaching themselves from the under surface of the general mass of this plate. The lower layer of these cells is in intimate contact with the thickened entoderm below. One or more superficial primitive grooves

may be observed in the surface layer of cells. Cells shortly begin to move downward in the median line from the area of the epiblast at the anterior edge of the caudal plate. These newly involuting cells, assisted by mitosis, move forward in the median line below the overlying epiblast. That is, the direction of cell movement is downward and forward, although its movement forward is not extensive. This is the main direction of motion. A pronounced lateral movement of cells is lacking. These activities, i.e., involution and mitoses, have as their main objective the construction of the substance of the streak or blastoporal area in a strict sense and in doing so to intimately contact the lower-lying entoderm. Now involutions of cells associated with mitosis occur in front of this beginning streak with the same apparent downward and forward movement of cells in the median line. In other words, gastrulation as such is not an active function of early streak development but rather these activities constitute a pregastrulation movement designed to develop and define the exact edges of the developing blastopore on the one hand, and on the other, to form a contiguous relationship between presumptive mesoderm and entoderm. This contact is present at the beginning of gastrulation in all lower chordates.

The fact that there is little lateral movement of cells from the sides of the streak during its earlier stages was carefully shown by the reconstruction studies and photomicrographs presented by Wetzel, '29, on the developing streak of the chick embryo. Also, Chen, '32, in observational studies on the duck blastoderm came to a similar opinion (pp. 157 and 158). As the streak grows from its beginning stages into the middle, half-way or broad streak (about stages 3-3+ of Hamburger and Hamilton, '51) a few, scattered, free mesodermal cells do appear on either side of the streak. The distance travelled by these cells from the body of the streak is small and in some regions along the streak they appear to be absent on one side but present on the other. These relatively few detached "free mesoderm cells", as Wetzel called them, undergo mitosis and in this way add to their bulk. These cells are incidental to the forward growth of the streak. In fact, in view of the activities of cell division and the downward and forward involution of cells which appear to be the processes by which the streak's substance is deposited, it would be difficult to eliminate a few cells from straggling outward along the sides of the streak.

In some streaks, when the half-way or broad streak stage is reached there are a number of free cells which appear to be arising from the sides of the posterior end of the streak and from the caudal plate. These cells extend outward toward the area opaca. They form part of the future extra-embryonic mesoderm. In consequence, the only objective conclusion that one can arrive at as far as early streak development to the half-way or broad condition is concerned is that the main morphogenic forces are at work delineating the immediate site of the streak (blastopore) and bringing the forming streak's substance into intimate contact with the entoderm below.

At about the time that the first quarter of the streak has been established there appears a tongue-like mass of free mesoderm at the anterior end of the streak. Wetzel, '29, called these cells the "anterior free mesoderm" (vorderes freies mesoderm) while Adelmann, '22, and Chen, '32, referred to them as the "pre-axial mesoderm." The anterior free cells comprise a forward growth or proliferation of cells from the anterior end of the developing streak which appears to be probing, as it were, the entodermal area in front of the streak. At the broad or half-streak stage they are prominent and form a mass of cells saddled upon the entoderm just anterior to the cephalic tip of the streak. It has been shown by Bellairs, '53a, that in the long or definitive-streak stage, the presumptive entoderm of the foregut comprises the entoderm which lies around and slightly forward from Hensen's node. The anterior free mesoderm at this stage, as in the half-way or broad streak, lies upon the presumptive foregut entoderm, and it represents, in part, the future pre-chordal plate material as observed by Adelmann, '22. In the broad streak stage the anterior end of the streak, and the anterior free mesoderm, approach the middle of the area pellucida. As the broad streak transforms into the slender, definitive streak (Stage 4 of Hamburger and Hamilton, '51), the area pellucida enlarges posteriorly to form the neck of the pear-shaped pellucid area. As Spratt, '46, pointed out, the posterior part of the streak increases greatly in length during this transformation period. The anterior end of the streak, and presumably the entoderm below, appears to be relatively stabilized during this transformation period. This suggests that once the anterior free mesoderm of the broad-streak stage approaches and comes in contact with the presumptive entoderm of the foregut, changes in the streak occur. Possibly this contact may initiate the transforma-

tion of the pregastrular blastopore or broad streak into the gastrulating blastopore or definitive streak. Be that as it may, accompanying the final aspects of this transformation into the definitive streak, cells move out increasingly from the area of Hensen's node to the differentiating dorsal region and the more lateral areas of the developing body, while extra-embryonic mesoderm travels distad from the posterior segment of the streak. The primitive groove before this time was shallow, if present at all, but now it deepens indicating vigorous invaginative tendencies accompanying cellular involution.

Certain information is available which may be used to support the above interpretation. Spratt, '46, p. 284, reported six cases in which the epiblast was isolated from the hypoblast in the pre-streak stage. Primitive streak development failed when these isolated epiblasts were cultured in vitro. On the other hand, Waddington, '32, p. 193, concluded "that a considerable amount of differentiation can take place in blastoderms from which the entoderm has been removed in middle or late primitive streak stages." Waddington added that it was "impossible to be certain that all of the endoderm" had been removed from the epiblast immediately below the primitive pit region. Also, Waddington, '33, was unable to induce the formation of new primitive streaks by epiblast rotation in relation to the hypoblast in older streaks but was successful in young streaks in which the area pellucida was still rounded in shape or nearly so. Dalton' 35, came to the conclusion that the removal of entoderm from the anterior end of the young streak stopped mesoderm differentiation. Fraser, '54, found the node entoderm and mesoderm intimately associated in the definitive streak. The removal of the node entoderm with damage to the associated mesoderm at this stage prevented further development or resulted in maldevelopment. Fraser concluded that "the endoderm at the node region was most necessary" for development "while the peripheral endoderm of embryos of this stage was not essential for development under this set of conditions". Nelsen, '58, observed that 4% O_2 was sufficient for early streak development to about the broad streak stage, but 6-8% O_2 was necessary for proper functioning of the definitive streak, particularly the nodal area. These observations, generally considered, support the interpretation that: 1. Entoderm is important in influencing the formation of the young streak, the influence continuing to about the broad streak stage; 2. mesodermal contact with the entoderm, particularly with the presumptive foregut ento-

derm, is important for gastrulation and early axial organization; 3. there are two main periods of streak development: a. the period of the young streak, culminating in about the broad streak condition, and b. the period of the maturing or transitional streak reaching its climax in the fully developed definitive streak; 4. there are activities involving the hypoblast and epiblast in the period of the young streak which are absent in the maturing streak, especially in its later stages.

b. *The streak and extra-embryonic tissue formation.* The formation of extra-embryonic tissues is a phenomenon pertaining to specialized, adaptive features which are not included within the scheme of the body in its final form. These adaptations enable the embryo to survive in its peculiar environment, and different environments call forth special responses even within a particular subdivision of a closely related animal group. For example, the formations of the extra-embryonic mesoderm in human and pig embryos are precocious affairs. In the human embryo the streak is almost entirely limited to the function of body-formation whereas in the pig the caudal area of the developing streak assumes the added responsibility of extra-embryonic mesoderm segregation. In the chick embryo the extra-embryonic mesoderm is segregated in a faltering manner from the posterior area of the streak at about the broad streak stage. As previously observed, there is considerable variation in intensity of this segregation among different blastoderms. Thus, there is a tendency toward precocity in the segregation of the extra-embryonic mesoderm in some chick blastoderms whereas in others the segregation is retarded toward the time when the definitive form of the streak assumes its functions.

It seems apparent that the ventral (posterior) lip of the functional blastopore (definitive primitive streak) in the chick embryo has been modified in its function to satisfy the demands of a special embryonic problem, namely, the segregation of extra-embryonic mesoderm, whereas in the pig embryo the caudal end of the early blastopore begins copiously to segregate extra-embryonic mesoderm to fill the requirements of a rapidly expanding blastocyst. The developing young blastopore of the pig, meanwhile, grows forward as in the chick to contact the hypoblast below. The situation in the human embryo is somewhat different due to the very precocious segregation of most of the extra-embryonic mesoderm presumably from the wall of the blastocyst, with only a small part segregated from the caudal end of the developing blastopore (streak). The developing blastopore in the hu-

man embryo thus has abandoned almost entirely use of its posterior lip as the main site of origin of extra-embryonic mesoderm. It appears from this view that the functional blastopore, i.e., the mature or definitive primitive streak, of chick, pig, and human embryos, with the exception of extra-embryonic tissue segregation from the posterior lip area, is homologous in general features with the blastopore of lower chordates. Strongly supporting this view is the behavior of the entoderm in the chick embryo as it migrates forward from the anterior edge of Hensen's node during the formation of the head process and beginning foregut. See Bellairs, '53b. It is to be particularly observed that the human embryo is more closely comparable in blastopore activity with lower chordates.

c. *The pregastrular movements in the bird embryo compared with similar movements in lower chordates.* The pregastrular movements of presumptive organ regions in lower chordates such as Styela, Amphioxus, frog, etc., occur within the egg's cytoplasm at fertilization and during the first cleavage. These movements involve particularly spatial changes in the position of presumptive entodermal and mesodermal cytoplasms. In the case of Styela (see Conklin, '05), presumptive mesodermal cytoplasm becomes aggregated at the future posterior-dorsal end of the egg (embryo) but gradually its crescent-shaped arms extend or progress in a dorso-anterior direction as the presumptive entoderm moves into a dorsal position. Finally, when the first cleavage plane occurs, the future notochordal cytoplasm appears in a cranio-dorsal position and the clear cytoplasm elongates to complete the presumptive blastoporal ring of mesodermal cyctoplasm. The presumptive blastopore thus lies in a dorsal position. Similar movements apparently occur in Amphioxus. (Conklin, '32 and '33.) The primitive position of the presumptive entoderm, chorda-mesoderm and blastoporal zone in these chordates is mainly a dorsal one with a slight infringement upon the caudal margin. As cleavage and blastulation occur, these areas continue to occupy the same relative positions established at fertilization.

In the bird embryo pregastrulation movements of the presumptive entodermal and chorda-mesodermal areas do not form a part of the fertilization activities, but are deferred until later. Lutz, '55, in the duck blastoderm (and also Patterson, '09, in the pigeon, to a certain extent) describes the movement of the entoderm from a dorsal position in the epiblast to a more ventral position at the margins of the

epiblast, with a concentration at the caudal margin from whence it moves forward. If this movement of the entoderm is eventually established for the bird blastoderm it would mean that the entoderm by this movement leaves its primitive dorsal position for a more ventral one. However, the evidence presented by Hunt, '37a and '37b, and by Fraser, '54, suggest that all of the presumptive entoderm does not leave its dorsal position in the epiblast in this manner. Forward movements of the hypoblast and epiblast just previous to primitive streak formation were described by Pasteels, '37a. These two simultaneous movements Pasteels called the "mouvements simultanés". During this period the hypoblast moves forward toward the anterior end of the area pellucida and thus establishes the didermic blastoderm. Within the epiblast the presumptive chorda-mesoderm pushes anteriad from a posterior position in the caudal region of the area pellucida; it becomes concentrated along the midline in the posterior half of the pellucid area. Spratt, '46, also described a forward movement in the hypoblast just previous to primitive streak formation and suggests that "the primitive streak depends for its origin and direction of development upon some stimulus originating in the hypoblast". The experiments of Waddington, '32, referred to above may be used to support this point of view. However, Waddington mentions the hypoblast as an influence in effecting the form-building movements in the epiblast necessary for streak development but does not imply that the actual formation of the streak is thus activated. The position taken by the present author relative to the pregastrular movements and primitive streak formation is that the movement in the epiblast should be considered as continuing until the substance of the early streak is deposited as far as the broad streak stage, and intimate contact thereby established between the chorda-mesoderm and hypoblast, particularly with its presumptive foregut region. That is, as the mesoderm cells of the epiblast come together in the median plane from either side they have a tendency to pile up in this plane. This piling up was suggested by Graper, '29, Wetzel, '29, Pasteels, '37, Malan, '53. As the cells aggregate in the midline they begin to involute and in consequence migrate slowly downward and to some extent forward in the midline to contact the entoderm below.

Malan (op. cit.) recognized the intimate association of the pregastrulation movements (called by Malan the "pre-invagination movements") with the deposition of early primitive streak substance.

Malan wrote concerning the pre-invagination movement in the epiblast that "although it precedes the actual invagination it is closely and progressively followed by the latter, so that the two types of movement take place simultaneously". P. 175. It is this simultaneity of movement together with the absence of marked lateral migration of cells from the lateral aspects of the forming streak which affords the basis for a view that the formation of the streak's substance to about the broad streak stage is a part of the pregastrulation phenomena. However, Malan emphasized "that the two types of movement are quite distinct and independent of each other". She believed that "it is more than probable that the invagination can proceed apparently quite normally even when the pre-invagination movements are almost totally inhibited under certain experimental conditions". P. 176. At this point in her expression allusion is made to the work of Spratt, '46, and Pasteels, '53, to substantiate the idea that formation of the streak's substance can proceed when pregastrulation movement in the epiblast is almost totally blocked. It is important to point out that the work of these two experimental studies supports the idea that under explant conditions some movement of cells in the epiblast toward the midline occurs. As a result, a piling up of cells in the midline with the subsequent involution movement which deposits cells to form the substance of the developing streak also is present. In those cases where Pasteels obtained weak inhibition of the pregastrular movements the streak was well developed; where it was strong, primitive streak formation was weak. The evidence to date does not warrant a conclusion that these two movements are distinct.

It appears, therefore, that relatively close comparisons are possible between the pregastrular activities of the more primitive chordates and those of the chick blastoderm. In the first place, in both types of embryonic development the presumptive mesodermal area extends or moves anteriad along the dorsal aspect of the future embryonic axis from a more posterior position as the different regions of the presumptive organ-forming materials are delineated. Secondly, the blastoporal lip or edge is defined in a dorsal position during the pregastrular movement of the mesoderm. Finally, although the entoderm in the chick blastoderm for the most part segregates away from its more primitive dorsal position, and comes eventually to form a lower, ventral layer of the didermic blastoderm, continuity between the

entoderm and mesoderm is re-established as the mesoderm extends forward by the deposition of the substance of the developing primitive streak. Intimate contact between the yolk-entoderm complex and the presumptive mesoderm appears necessary for gastrulation in the chordate series. (See also, Vintemberger, '36.)

Apropos of these considerations, it is to be noted that Pasteels, '37b, found that the presumptive entoderm in the turtle, Clemmys leprosa, remains in its primitive dorsal position surrounded by a ring of future mesoderm until gastrulation is about to begin. At this time the entoderm hastily migrates inward ahead of the mesoderm to establish the hypoblastic layer of the didermic blastoderm.

Summary

1. Increased partial pressure of O_2 to the extent of 1/5 atmosphere in the chick embryo elicits an accelerative response in development during the late primitive streak stage and stages immediately following.

2. Increased partial pressure of O_2 to the extent of 1 atmosphere increases the rate of development in the frog embryo at about the time gastrulation begins but not during cleavage and blastulation.

3. Evidence is presented to support the concept that the earlier phases of primitive streak development represent, in reality, a portion of the ensemble of pregastrulation movements of presumptive organ-forming areas, and that gastrulation begins at the dorsal blastoporal lip region (anterior end of primitive streak) as the definitive, organized form of the streak is established (see also, Nelsen, '57).

4. The primitive streak may be considered as experiencing three general periods of development, namely, 1. a pregastrular period, terminating at about the broad streak stage, i.e., at about stages 3 to 3+ of Hamburger and Hamilton, '51; 2. a transitional or intermediate period which ends in 3. the gastrular, long, or definitive streak, i.e., Hamburger and Hamilton, '51, stage 4.

5. Probably one of the important prerequisites for the onset of definitive streak formation and the beginning of gastrulation is the contact made, at the broad streak stage, between the anterior free mesoderm (prechordal plate, in part) and the presumptive foregut entoderm.

6. It is concluded that the general response in the chick and frog embryos to increased partial pressures of O_2 indicate homologous periods of development.

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EFFECT OF CERTAIN FATTY ACIDS ON ROUS SARCOMA VIRUS*

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IN OUR previous paper (14), we reported isolation of a substance, the product of enzymic activity of *Penicillium brevi-compactum*, which inhibited influenza virus PR8 and Rous sarcoma virus in chick embryos. This substance, easily oxidized and protein-free, considerably reduced the number of lesions, as compared with control, and prevented the formation of tumors. The inhibitory effect of this substance on Rous virus was not, however, complete. In view of the important role which lipids apparently play in the propagation and infectivity of this virus, an attempt was made to prepare active factors from fatty acids.

Pollard (12) has estimated that the lipid content of the microsome fraction of Rous tumor was as high as 53 per cent of the total dry-weight substance. Claude (3) suggested that the lipid fraction of a Rous tumor microsome preparation plays a part in inactivation of the tumor inducing agent. Wilbur *et al.* (19) have demonstrated that oxidized unsaturated fatty acid esters inhibited cell division. According to Ottolenghi *et al.* (10) and Bernheim *et al.* (2), oxidized methyl linolenate and oxidized methyl linoleate inhibited mitochondrial enzyme activity. Fisher and Wilbur (4) disclosed that oxidized methyl linolenate depolymerized DNA. Shuster (13) has demonstrated that the oxidation products of unsaturated fatty acid esters inhibited respiration and glycolysis of Ehrlich ascites tumor. Tsukada and Fu (17) found that respiration of Ehrlich ascites tumor was inhibited strongly by caprylate. Groupé *et al.* (5) disclosed that sodium oleate had

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virucidal activity against vaccinia virus. Begg and Aitken (1) found that the application of sodium oleate occasionally caused regression of Rous tumor. Toyomizu *et al.* (16) pointed out, on the basis of their observations, that the unsaturation of fatty acids seemed to be the factor responsible for the biologic activity of fatty acid fractions. Moloney (8) has shown that the tumor-producing activity of Rous virus was inhibited by the oxidation products of Rous sarcoma lecithin and microsome lipids and by methyl linolenate. Helmer and Clowes (6) have obtained some retardation of the tumor growth by administering the lipid fraction of pig pancreas to chicks, bearers of Rous sarcoma. Pirie (11), described the effect of pancreas extracts on the Fujinami tumor virus and attributed inhibition of virus activity to fatty acids present in the extract. The recent work of Youngner and Noll (20) demonstrated that viruses are absorbed by lipids.

In our present study, a number of unsaturated fatty acids, oxidized and unoxidized, as well as the quaternary salts, were tested for their inhibitory activity against Rous virus by the chick embryo technique and by inactivation *in vitro*.

Material

The following saturated and unsaturated fatty acids were tested on Rous virus: Caproic, undecylic, myristic, palmitic, oleic, undecylenic, linoleic, linolenic acids; methyl undecylenate, isopropyl undecylenate, methyl linoleate, and methyl linolenate. Also unsaturated fatty acid fractions of pilchard oil (i.v. 287.2); of cod liver oil (i.v. 248.8); of menhaden oil (i.v. 244.7); of safflower oil (i.v. 85.3); ethyl ester of unsaturated fatty acids, pilchard oil (i.v. 267.1); cod liver oil (i.v. 269.9); and safflower oil (i.v. 148.6).

Six quaternary compounds were prepared: tetradecyltriethylammonium bromide; hexadecyltriethylammonium bromide; octadecyltriethylammonium bromide; tetradecylpyridinium bromide; hexadecylpyridinium bromide and octadecylpyridinium bromide.

Hexadecyltriethylammonium bromide was prepared by heating bromohexadecane with triethylamine in ethyl alcohol for 3 hours at 95° C. Hexadecylpyridinium bromide was prepared by heating bromohexadecane with pyridine for 1.5 hours at 150° C. By these methods, two triethylammonium bromides and two pyridinium bromides were prepared from bromotetradecane and bromooctadecane.

Fatty acids were oxidized by means of cupric stearate as a catalyst

and oxygen. The iodine value of every compound tested was checked according to the Official Method, A.C.C.S., Da 12-48, Section C. The thiobarbituric acid (TBA) reaction was used to determine the degree of fatty acid oxidation. The procedure was approximately the same as recommended by Wilbur *et al.* (18).

Methods of Testing

As the first step in our study, all the fatty acid compounds and fractions were tested on mouse influenza virus PR8, the egg adapted strain, in chick embryos. This testing gave us an indication about the maximal non-toxic dose of the material and its antiviral activity, if any. Influenza virus PR8, 0.1 ml., was inoculated into the chorioallantoic cavity of an eleven-day-old embryo, and fifteen minutes later, the material in 0.1 ml. distilled water solution or suspension was injected into the same cavity. The virus was titrated and the ID 50 estimated by the Salk pattern method. Duration of incubation after inoculation: 24 hours. The suspension of water-insoluble compounds and fractions were prepared by means of tween 20 and propylene glycol. The following formula was used: 75% distilled water, 16% tween 20 and 9% of propylene glycol. This vehicle, when tested, showed neither toxicity to chick embryos, nor any inhibitory activity on the virus. These tests indicated that 1.0 mg/0.1 ml. of fatty acids was not toxic to chick embryos, while 2.0 mg/0.1 already showed some toxicity. The quaternaries, 0.03 mg/0.1 ml. were not toxic, but at a dose of 0.04 mg/0.1 ml. they manifested certain toxicity. On the basis of these preliminary findings, all the compounds and fractions of fatty acids were tested on Rous sarcoma virus by the chick embryo technique. The Rous sarcoma virus, obtained from the American Type Culture Collection was diluted x 100, in Simms salt solution, to which 1% Bovine albumin (Armour Laboratories) was added. The diluted virus, 0.1 ml., was inoculated onto the chorioallantoic membrane of a ten-day-old chick embryo. Each test covered 75 eggs, twenty-five serving as control and fifty eggs used for two samples of fatty acids. The material to be tested was injected, 1.0 mg/0.1 ml./egg, onto the membrane, fifteen minutes after virus inoculation. In the case of the quaternaries, the inoculum was 0.03 mg/0.1 ml./egg. On the seventh day after the inoculation, the eggs were opened, and the number of lesions and their size were recorded.

These experiments were supplemented by testing the material by

inactivation *in vitro,* for which the technique of Moloney (8), slightly modified, was used. The suspensions of water insoluble fatty acids, 2 mg/0.1 ml. were prepared by means of tween 20 and propylene glycol, with phosphate buffer, pH 6.2. The water soluble quaternaries were used in concentration 0.05 mg/1.0 ml. in distilled water. To 4.5 ml. of the standard Rous virus, diluted x 100, pH 6.6, we added 0.5 ml. of a fatty acid suspension or of a quaternary solution. Thus the samples to be tested had the concentrations 0.2 mg/1.0 ml. for water insoluble fatty acids, and 0.005 mg/1.0 ml. for water soluble quaternaries. The samples were incubated at 37° C for 90 minutes, as was the control sample of Rous virus. The tumor-producing property of the control sample was taken to be one hundred per cent. After the incubation, the samples of fatty acids, and of the control were inoculated subcutaneously into the wing web of 20-day-old chicks, in the amount of 0.2 ml. The inactivation of Rous virus by various fatty acids was estimated on the basis of number of takes, and calculated as percentage of the one hundred per cent takes in the control group.

Results

The results of these tests are briefly summarized in Tables I and II. Table I refers to the data obtained with the chick embryo technique. Table II presents the data with the inactivation *in vitro.*

Analyzing the results of Table I, one may see that water insoluble fatty acids of various iodine value show no activity against Rous sarcoma virus. This fully corresponds to the data obtained with these compounds and fractions in testing on influenza virus PR8. Neither of them has manifested any significant antiviral activity. On the other hand, the quaternaries possessed a marked activity against both influenza virus and Rous virus at the inoculum 0.03 mg/0.1 ml./egg. Larger doses of these compounds were found to be toxic to the embryos. Among them the most active were hexadecyltriethylammonium bromide and hexadecylpyridinium bromide, both being saturated compounds. They reduced the number of Rous virus lesions by 50-60%. By preparing unsaturated alkyl pyridinium bromide and unsaturated alkyl triethylammonium bromide from fish oil fatty acids, we were able to obtain even more active compounds. The unsaturated alkyl pyridinium bromide, iodine value 147.2, inhibited the formation of Rous sarcoma lesions in chick embryo by 80 per cent as compared with control, at the virus dilution x 100. The unsaturated alkyl

TABLE I
THE EFFECT OF FATTY ACIDS ON ROUS VIRUS IN CHICK EMBRYO
Dilution of Rous Virus x 100, Inoculum 0.1 ml.

Compound	Inoculum in mg/0.1 ml.	Inhibition of Rous Tumor (*)
Caproic acid	1.0	—
Myristic acid	1.0	—
Palmitic acid	1.0	—
Oleic acid	1.0	—
Oleic acid, oxidized	1.0	±
Undecylenic acid	1.0	—
Undecylenic acid, oxidized	1.0	—
Linoleic acid	1.0	—
Linoleic acid, oxidized	1.0	±
Linolenic acid	1.0	—
Linolenic acid, oxidized	1.0	±
Methyl linolenate	1.0	—
Methyl linolenate, oxidized	1.0	+
Unsat. F.A., pilchard, i.v. 287.2	1.0	—
Unsat. F.A., pilchard, oxidized	1.0	±
Unsat. F.A., menhaden, i.v. 244.7	1.0	—
Unsat. F.A., menhaden, oxidized	1.0	—
Unsat. F.A., cod liver, i.v. 248.8	1.0	—
Unsat. F.A., cod liver, oxidized	1.0	±
Ethyl Ester F.A., pilchard, i.v. 267.1	1.0	—
Ethyl Ester F.A., pilchard, oxidized	1.0	—
Ethyl Ester F.A., cod liver, i.v. 269.9	1.0	—
Ethyl Ester F.A., cod-liver, oxidized	1.0	—
Ethyl Ester F.A., safflower, i.v. 148.6	1.0	—
Tetradecyltriethylammonium bromide	0.03	+
Hexadecyltriethylammonium bromide	0.03	+++
Octadecyltriethylammonium bromide	0.03	++
Tetradecylpyridinium bromide	0.03	+
Hexadecylpyridinium bromide	0.03	++++
Octadecylpyridinium bromide	0.03	++

(*) — No decrease in the number of lesions as compared with control.
± An average decrease of 5-10% in the number of lesions as compared with control.
+ An average decrease of 15%.
++ An average decrease of 30%.
+++ An average decrease of 50%.
++++ An average decrease of 70%.

The water-insoluble samples of fatty acids were emulsified by means of tween 20 and propylene glycol.

ammonium bromide, iodine value 120.9 showed almost identical activity of about 78 per cent inhibition. In both instances, the inoculum was 0.03 mg/0.1 ml. Apparently the unsaturation of quaternaries increased their biological activity.

Quite different results were obtained in our testing fatty acids by inactivation of Rous virus *in vitro* (Table II). Here, many fatty acid fractions and compounds induced inactivation of the virus at the

TABLE II

INACTIVATION OF ROUS SARCOMA VIRUS BY FATTY ACIDS

Inactivation is estimated as percentage of the activity of the control samples which were taken to be one hundred per cent.

Compound	Conc. in mg. per 1.0 ml.	% Inactivation
Caproic acid	0.2	None
Myristic acid	0.2	None
Palmitic acid	0.2	None
Oleic acid	0.2	None
Oleic acid, oxidized	0.2	10
Undecylenic acid	0.2	12
Undecylenic acid, oxidized	0.2	26
Isopropyl undecylenate	0.2	None
Isopropyl undecylenate, oxidized	0.2	None
Linoleic acid	0.2	9
Linoleic acid, oxidized	0.2	22
Linolenic acid	0.2	23
Linolenic acid, oxidized	0.2	50
Methyl linoleate	0.2	14
Methyl linoleate, oxidized	0.2	28
Methyl linolenate	0.2	26
Methyl linolenate, oxidized	0.2	60
Unsat. F.A., pilchard, i.v. 287.2	0.2	15
Unsat. F.A., pilchard, oxidized	0.2	21
Unsat. F.A., menhaden, i.v. 244.7	0.2	10
Unsat. F.A., menhaden, oxidized	0.2	22
Unsat. F.A., cod liver, i.v. 248.8	0.2	15
Unsat. F.A., cod liver, oxidized	0.2	22
Unsat. F.A., safflower, i.v. 85.3	0.2	Almost None
Unsat. F.A., safflower, oxidized	0.2	Almost None
Ethyl Ester F.A., pilchard, i.v. 267.1	0.2	None
Ethyl Ester F.A., pilchard, oxidized	0.2	10
Ethyl Ester F.A., cod liver, i.v. 269.9	0.2	None
Ethyl Ester F.A., cod liver, oxidized	0.2	10
Ethyl Ester F.A., safflower, i.v. 148.6	0.2	None
Ethyl Ester F.A., safflower, oxidized	0.2	None
Tetradecyltriethylammonium bromide	0.005	25
Hexadecyltriethylammonium bromide	0.005	77
Octadecyltriethylammonium bromide	0.005	51
Tetradecylpyridinium bromide	0.005	27
Hexadecylpyridinium bromide	0.005	79
Octadecylpyridinium bromide	0.005	53

concentration of 0.2 mg/1.0 ml. The quaternaries inactived Rous virus in a very small concentration, 0.005 mg/1.0 ml. following the same pattern as was recorded in the tests in chick embryo. There was no apparent discrepancy between the two tests as far as the quaternaries were concerned.

The difference in the end results of the two tests was also evident with oxidized fatty acids. In our testing on influenza virus PR8

and Rous sarcoma virus by the chick embryo technique only oleic, linoleic, linolenic and unsaturated fatty acids from the pilchard and cod liver oils (of high iodine values) have shown a slight activity after oxidation. The rest of the fatty acids, when oxidized, were lacking all activity against the two viruses (Table I). In the tests with inactivation *in vitro,* however, many fatty acids manifested various degrees of activity against Rous virus (Table II).

Discussion

Our present study indicates that water insoluble fatty acids by themselves exert very little, if any, inhibitory activity against Rous sarcoma virus, when tested by the chick embryo technique. When, on the other hand, the same compounds and fractions were tested by the method of inactivation *in vitro,* a number of them inactivated the virus, the fact reported by several other investigators. One may assume that there were present some chemical or physical reactions in the tests *in vitro,* which were absent in the chick embryo testing. The nature of these reactions might find a proper interpretation in the findings of Younger and Noll (20) concerning the lipid-virus interaction.

Oxidation of highly unsaturated fatty acids resulted in the appearance of a moderate inhibitory activity against Rous virus as evidenced by the chick embryo testing. It was much more pronounced in the tests with inactivation *in vitro.* The chemical nature of this phenomenon was discussed by several investigators (Moloney (8), Ottolenghi *et al.* (10) and others). It was pointed out by them that in the course of the oxidizing process, a water-soluble fraction was formed, containing the oxidized products of fatty acids, peroxides or aldehydes; peroxides by themselves having some virucidal activity. Tappel (15), on the other hand, observed that unsaturated fatty acids could react with protein, forming rather stable complexes, which could not be ruptured by solvent treatments. He attributed the stability of these complexes as being caused by a chemical union between aldehydes produced during auto-oxidation of the lipids and the reactive amino groups of the protein. Narayan and Kummerow (9) have demonstrated experimentally this affinity of aldehydes for proteins. They obtained the complexes formed between egg albumin and oxidized linoleic acid. Little or no complex formation could be obtained with unoxidized linoleic or lauric acids.

The remarkable inhibitory activity of unsaturated quaternaries of fatty acids prepared from fish oils on Rous sarcoma virus depends probably not only on their water-solubility but on their chemical structure.

The tumor-producing viruses, like Rous chick sarcoma virus or the chick lymphomatosis virus, form a separate group of viruses. Not only their property to penetrate into the cells seems to be greater than that of many other viruses, as shown by our cytologic studies of the chick embryos receiving intravenous injection of Rous virus, but their peculiar property of inciting tumor growth is very pronounced. In our experiments with our antiviral factor A-14, a product of *Penicillium brevicompactum* fermentation, we were unable to inhibit completely Rous virus either in chick embryo or in baby chicks. In about 20% of embryos and in 35% of baby chicks, the Rous sarcoma developed in spite of the treatment. Even less satisfactory results were obtained with the chicks inoculated with the Strain RPL-12 virus of the intravascular lymphomatosis supplied by Dr. Ben Burmester. The mortality rate of the chicks was reduced by only 40-50% in spite of an intensive treatment with this factor. By combining the factor A-14 with the unsaturated quaternaries hexadecyltriethylammonium bromide or hexadecylpyridinium bromide we were able to increase the inhibition of Rous virus up to 88-90%. Yet some part of the virus always evaded the effect of the antiviral factors. Only by combining the A-14 factor with Mitomycin-C, the newly isolated antibiotic discovered and produced by Kyowa Fermentation Industries, Tokyo, Japan, possessing a strong antitumor property, we succeeded to achieve a complete inhibition of Rous virus in the chick embryo tests. These results, which will be reported in our forthcoming paper, seem to indicate that the attack on the tumor-producing viruses should be conducted by combining both antiviral and antitumor factors.

Summary

Fourteen fatty acid compounds and fractions, and six saturated and six unsaturated quaternaries were tested for their activity against Rous virus by the chick embryo technique and by inactivation *in vitro*.

The fourteen water-insoluble fatty acids showed no inhibition of Rous virus in chick embryo. Eight of them inactivated the virus *in vitro* in various degrees.

Both the saturated and unsaturated quaternaries exerted a strong inhibition of the virus in chick embryo and inactivated it *in vitro*.

Oxidation of oleic, linoleic, linolenic and the fatty acids from cod liver oil, iodine value 248.8, resulted in a slight inhibitory effect on Rous virus in chick embryo.

Oxidation of all highly unsaturated fatty acids tested resulted in inactivation of the virus *in vitro* in various degrees.

The discrepancy in the results obtained by the two tests was discussed.

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ELIMINATION OF PLASMA IN CULTURING HUMAN NEOPLASTIC TISSUE

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IT IS well known that the use of plasma in tissue culture technique although valuable for obtaining cell growth, is often found to be disadvantageous since plasma clots may become cloudy or liquified.

For a number of years the writer has eliminated plasma in the cultivation of human neoplastic tumors, and replaced it by a mixture of equal parts of Amigen solution and honey. The Amigen solution is prepared by Mead Johnson and Company (1) and is easily obtained in any hospital. It is made up of 5% Amigen (a pancreatic hydrolysate of casein consisting of amino acids and small peptides) and 5% Dextrose. Honey is mainly a mixture of three sugars: Levulose, Dextrose and Sucrose. The Amigen and Honey mixture was employed in the same way as the plasma which is used in the usual tissue culture technique. From a capillary pipette two drops of the mixture are placed on the center of a coverslip (coverslip technique); the tissue fragments to be cultivated are then introduced into the center of the medium. The coverslip with the mixture and the tissue fragments is then introduced into a Porter flask and after incubation at 37°C for 15 minutes, the usual tissue culture nutrient medium is added.

The most important results of the elimination of plasma and substitution of Amigen and Honey mixture are: 1) a much clearer picture of the cells and tissues to be studied can be obtained, 2) the tissues and cells cultured keep their characteristic appearance and growth patterns even after a long period of cultivation.

Figure 1 illustrates a 6-day-old living culture from a brain tumor at a magnification of 430 times, which was diagnosed *in vitro* as a glioblastoma multiforme. The growth was rapid, there was a lack of glial fiber formation; cellular debris, vascular coils and mitotic

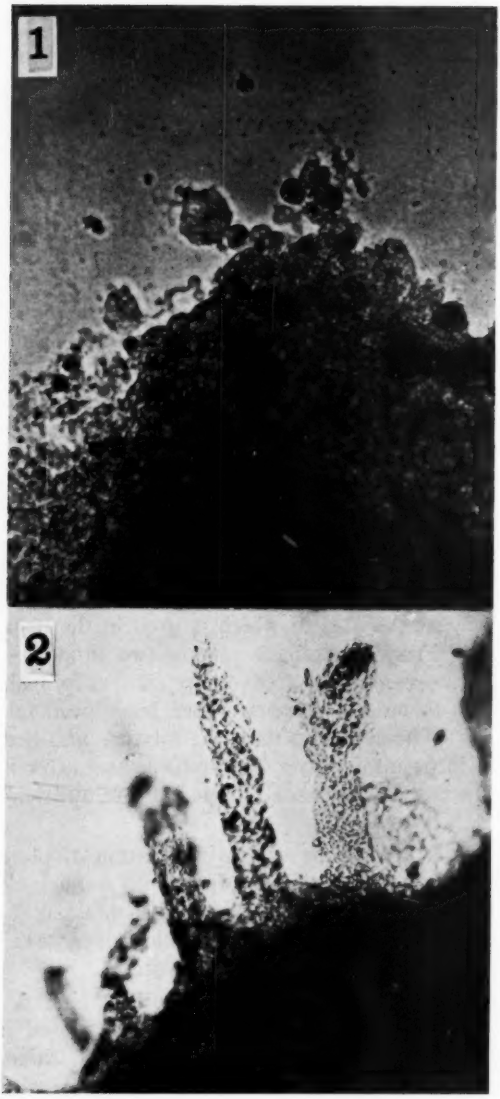

Figure 1. Living culture of Glioblastoma Multiforme
Figure 2. Living culture of Carcinoma of Cervix

figures were observed. Among the striking cellular elements was the spongioblast containing spheroid inclusions.

Figure 2 shows a 48-hour living culture of carcinoma of the cervix at a magnification of 210 times. The extensive epithelial tongues growing rapidly during the first 24-48 hours are well defined.

The studies to date made on the cultivation of human neoplastic tumors, indicate that plasma is not necessary for the successful culturing of cells and tissues and that the above mentioned medium can be used as a substitute for plasma.

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1. Mead Johnson & Company. Evansville 21, Indiana.
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Growth, 1958, **22**, 229.

BOOK REVIEW

Bibliography on the Effects of Ionizing Radiations on Plants. Compiled by Arnold H. Sparrow, John P. Binnington and Virginia Pond—Brookhaven National Laboratory, Upton, N. Y., 222 pages, 1958.

In addition to the 2,575 references to research papers concerned with the effects of ionizing radiations on plants (omitting bacteria) arranged alphabetically according to authors, there are thirty-two references to published records of scientific conferences, congresses and symposia which pertain to this subject. About 600 periodicals in which the papers have been published are listed along with their abbreviations as used by the American Chemical Society and, finally, an exhaustive subject index has been compiled, largely from words used in the titles of the research papers. References to abstracts follow immediately those to the original papers in many cases. This bibliography with its accompanying sections, its excellent format and apparent completeness should be of value to investigators in the field of ionizing radiations and their effects on living organisms.

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